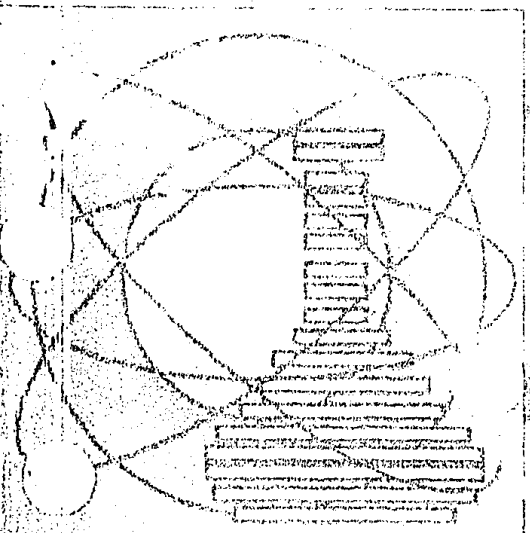

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OUR COVER

The cover, taken from the first manuscript published in this issue, is a photomicrograph of lymphocytes stimulated by B. thuringiensis subsp. morrisoni (Serotype H 8a:8b), PG-14 lectin.

The Philippine Journal of Science is a journal on basic sciences published quarterly by the Science and Technology Information Institute - Department of Science and Technololgy (STII-DOST) with editorial office in Bicutan, Taguig, Metro Manila.
Tel. no.: 837-2191 to 95 local 6/7
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THE PHILIPPINE JOURNAL OF SCIENCE

Jan. - Mar. 1997 Vol. 126 No. 1

ISOLATION AND CHARACTERIZATION OF A LECTIN FROM *Bacillus thuringiensis* subs. *MORRISONI* (SEROTYPE H8a:8b) PG-14

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ABSTRACT

A lectin which is mitogenic against human peripheral lymphocytes and has relatively weak mosquitocidal activity was isolated from the spores/crystals of B thuringiensis subsp. morrisoni (Serotype H 8a: 8b) PG-14.

The lectin was isolated by solubilizing the spores/crystals in alkaline pH in the presence of dithiothreitol. Purification was accomplished by ammonium sulfate fractionation and gel chromatography using Sephadex G-200. The purified lectin was found to be homogeneous by polyacrylamide gel electrophoresis under non-denaturing condition at pH 8.8.

The lectin was found to have no blood group and blood type specificity since it agglutinated all human blood types (A, B and O) and animal blood (calf and goat) used in the experiment. Hapten inhibition test did not show any sugar specificity based from the standard sugars used. The purified lectin is a glycoprotein with a total sugar content of 10.96% as determined by the phenol sulfuric acid method. The approximate molecular weight of the single polypeptide band obtained from sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was calculated to be 65 kD. Morphological examination of human lymphocyte cultured in the lectin showed that 43% of the cell population had been transformed after 72 hours indicating the mitogenicity of the lectin. Insect toxicity assay using 3-day old Aedes albopictus larvae resulted to a relatively lower mortality rate after 24- hr exposure to the lectin compared to the crude extract.

INTRODUCTION

Stillmark's discovery of a hemagglutinin from the seeds of *Ricinus communis* in 1888, is considered as the beginning of lectinology. Microbial lectins were discovered several years later. The first report on a fungal hemagglutinin was that of Kolbert in 1991 and of a bacterial hemagglutinin in 1902 (Liener, 1976).

The numerous observations on the microbial hemagglutinins were not correlated with those of plants, even when Boyd and Shapleigh coined the term for the latter, the microbial hemagglutinins were not included in it (Boyd and Shapleigh, 1954). In 1975, the first report on inhibition of bacterial hemagglutinin by a simple sugar was reported by Collier and de Miranda (1955) wherein D-mannose strongly inhibited the hemagglutinating activity of *E. coli*. Moreover, they stated that the occurrence of hemagglutinating activity in many pathogenic bacteria suggests that the pertinent adhesive properties may have their natural functions in fixing the bacteria or their toxins, to the surface of host cells.

Several works have shown that bacteria produce lectins specific for certain carbohydrates and the bacteria depend on those lectins for adhering to a host's tissue as the first step in the process of infection (Sharon & Lis, 1993). A toxin from *B. thuringiensis* reported to be glycopotein in nature (Holmes & Monro, 1965; Baeson & Stainby, 1979, Bulla et al., 1977, 1979; Huber et al., 1981, Tyler et al., 1981; Insell & Fitz-James, 1985) was reported to be toxic against lepidoptera insects (Tojo, 1986; Whiteley & Schnepf, 1986; Padua et al. 1980, 1984). The toxin has been shown to be associated with the crystalline inclusion of *B. thuringiensis* which is formed concomitantly with sporulation (Young & Fitz-James, 1959, Delafield et al., 1968). The crystal protein that is produced with every spore of *B. thuringiensis*, is an inactive protoxin, reportedly composed of molecules of mass 130 kD. It is activated by alkaline dissolution and breakdown by gut proteases into active toxins varying in molecular mass from 60-65 kD (Calabrese et. al., 1980 and Ghosh-Dastidar and Nickerson, 1979)

On the other hand, a proteinase-resistant protein was purified from *B. thuringiensis* subsp. *israelensis* which exhibited toxicity to mosquitoes' larvae and cultured mosquito cells, lysed erythrocytes and was lethal to mice. The protein was extracted from the sporulating culture of *B. thuringiensis subsp. israelensis* by treating with alkali, neutralized and incubated with trypsin and proteinase K & purified by gel filtration & DEAE column chromatography. The 25 kD fragment of the toxic protein was found to be responsible for the insecticidal, cytolytic, hemolytic and mouse-lethal activities of the crude toxin extract (Armstrong et.al., 1985; Davidson & Yamamoto, 1984).

Based on the reported glycoprotein nature of the toxin from *B. thuringiensis*, agglutination assay of the crude spores/crystals of several *B. thuringiensis* isolates were done to see whether the spore/crystal contain a lectin. Preliminary results revealed that different isolates have different degrees of lectin activity. Isolating and characterizing the lectin therefore could lead to a better understanding of the biochemical basis for the toxicity of this crystal protein against lepidopteran insects, and to a more effective control of insects for further improvement and development of *B. thuringiensis* as a microbial insecticide.

MATERIALS AND METHODS

Isolation of the Lectin

B. thuringiensis culture was obtained from Dr. Leodegario E. Padua of the National Institute of Biotechnology and Applied Microbiology and designated as APP1. The microorganism was maintained on a nutrient agar slant at 35°C. Cells for experimental use were cultured in a modified liquid Glucose-Yeast extract salt (GYS) medium at 28°C in a 2L-Erlenmeyer flask and was aerated by rotary agitation at 250 rpm for 72 hrs. Culture was held for another 5 hours after sporulation to allow individual cells to lyse and release spore and parasporal crystals.

The spores and parasporal crystals were removed from the medium by centrifugation (900 rpm, 15 minutes) and washed with NaCl, then 5X with distilled water and lyophilized. The crystal was solubilized by suspending in distilled water at 70°C for 1 hr and centrifuged. The pellet was resuspended in 0.1M glycine-NaOH (pH 9.5) at 30°C for 1 hr. Solid $(NH_4)_2SO_4$ was added to give 60% saturation to the supernatant obtained by centrifugation. The solid obtained by centrifugation was resuspended in 0.1M glycine-NaOH at 30°C and dithiothreitol (DTT) was added and centrifuged. The supernatant was collected and labelled as solubilized crystal protein and assayed for agglutination. The solubilized crystal protein was further purified in a gel chromatography column (2.5 x 20 cm). The Sephadex G-200 column with a bed volume of 70 ml was equilibrated with 0.05M Na_2CO_3 (pH 9.5) at a flow rate of 1 mL/min. Fractions were monitored for absorbance at 280 nm and assayed for agglutination.

Agglutination Assay

Human blood types A, B & O from Laguna Red Cross in Sta Cruz and animal blood from goat and calf from the Dairy Training Research Institute (DTRI) were used in the agglutination assay. The test was done in multiwell-microtiter plates using slightly outdated trypsinized and untrypsinized erythrocytes (Meimeth et al. 1982).

A 50 uL sample was serially diluted with 50 uL phosphate buffered saline (PBS) solution, then 50 uL of the 2% (v/v) erythrocyte suspension was added to each well. The plates were incubated for 1 hr at room temperature and examined visually.

Formation of a uniform layer over the surface of the well indicated a positive result while discrete button formation at the bottom of the well indicated a negative result. Titer values were taken as the reciprocal of the highest dilution with visible agglutination while agglutination titer was reported as the least concentration of lectin needed to cause agglutination.

Inclusion of the following sugars at 1000 mM and 200 mM concentrations in the agglutination assay will determine the sugar specificity of the lectin sample (Fountain & Campbell, 1984).

D-glucose	D-fructose	D-galactose
D-fucose	D-Xylose	D-arabinose
D-mannose	Lactose	Melibiose
D-rhamnose	Sucrose	N-acetylgalactosamine
Maltose	Cellobiose	N-acetylglucosamine
Raffinose	Methyl α-D-Mannopyranoside	

Characterization of the Lectin Isolate

Protein Determination

Total soluble protein of the solubilized crystal protein and the purified lectin fraction from gel chromatography were determined by the modified method of Lowry et al (1951) with bovine serum albumin (BSA) as standard.

Polyacrylamide Gel Electrophoresis (PAGE)

Non-denaturing condition

Electrophoresis in a discontinuous polyacrylamide slab gel system consisting of 4% polyacrylamide stacking gel in Tris-HCl buffer at pH 8.8 was conducted following the method of Laemmli (1970).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The molecular weight of the purified lectin isolate was estimated using sodium dodecyl sulfate (SDS) in PAGE following the procedure of Laemmli (1970).

The following protein standards were used

Standard Protein	MW	
Bovine albumin	66,000	
Egg albumin		45,000
Glyceraldehyde 3-phosphate dehydrogenase	36,000	
Carbonic anhydrase		36,000
Trypsinogen		24,000
Trypsin inhibitor	20,100	
α-lactalbumin		14,200

Carbohydrate Analysis

The total carbohydrate content of the purified lectin was determined by the phenol-sulfuric acid method of Duboir et al (1956) using D-glucose as standard.

The sugars were identified using a modified AOAC procedure (Dunwire & Otto, 1979) for glycoprotein and using High Pressure Liquid Chromatography (HPLC) Waters Associate.

Determination of Mitogenic Activity

The procedure of Toyoshima et al (1970) was followed in culturing human peripheral lymphocytes. A leukocyte-rich plasma was obtained from normal human blood type O. To a sterile culture tube containing 0.75 ml of NCTC-109 (Sigma), 0.15 ml of calf serum and 0.15 mL of sample solution (5 mg/mL), 0.45 mL aliquot of leukocyte-rich plasma was transferred. The cultures were incubated at 37°C for 72 hours. A positive control and a negative control were included in the experiment. The percent transformed cells was determined from Giemsa-stained preparation.

Insect Toxicity Assay

Second instar larvae of *A. albopictus* from BIOTECH were exposed to 7.5 ml of test solutions using 3 replicates per sample. Observations were recorded after 1 hr, 2 hrs, 3 hrs, 72 hrs, 96 hrs and 120 hrs.

RESULTS AND DISCUSSION

Isolation and Purification of BT lectin

Partial purification was done by solubilization of the crude spores/crystals at alkaline pH (pH 9.5) in the presence of dithiothreitol and by ammonium sulfate fractionation. In all cases, solubilization was done by swelling the crystals in buffer-containing denaturing agent or at pH above 9 and eventually solubilization of the crystals after addition of disulfide cleaving reagents (Huber et al., 1981).

Fractionation with ammonium sulfate at 0-60% saturation precipitated majority of the protein in the mixture. Further purification was accomplished by gel chromatography on Sephadex G-200 (Fig. 1). Two well-separated peaks were obtained one of which contain the lectin (Peak 1). The second peak contain the non-lectin protein component. Fractions from separate peaks were pooled, concentrated by polyethylene glycol (PEG) and lyophilized. The results of the purification steps are presented in Table 1. The specific activity was found to increase with purification.

The homogeneity of the isolated lectin was determined by polyacrylamide gel electrophoresis under non-denaturing condition. The final preparation of the purified lectin appeared homogeneous as indicated by a single band (Fig. 2) compared with the crude and solubilized crystal preparation.

Agglutination Assay

The purified lectin was found to be nonspecific because it agglutinated all types of human blood (A, B, O) and animal blood (calf & goat) used in the experiment. This suggests the existence of multiple binding sites on the lectin. Agglutination is relatively higher using human blood compared to using animal blood suggesting a weaker ability of the lectin to form bridges between cells and possibly fewer receptor sites for animal blood. Trypsination of the red blood cells increased agglutinability of the lectin possibly by the exposure of additional lectin-receptor sites which are previously in "cryptic" form (Table 2). Nicolson (1971) suggested a possible rearrangement of pre-existing sites.

No inhibition of agglutination was observed even at a high concentration of sugars used. Results of the hapten inhibition are summarized in Table 3. The results obtained may be explained by some structural features which may not be present in the purified lectin. According to Osawa (1966), the following are the structural features needed for sugar inhibition: (1) a non-reducing end sugar residue that is linked to the remainder of the molecule by a β-glycosidic linkage; (2) a glucosidic linkage of the non-reducing end sugar residue that is linked to a ring carbon atom of the next sugar residue or a benzene ring without interposition of a methylene group; (3) a non-reducing end sugar residue that have an unsubstituted hydroxymethyl group.

Characterization of the Lectin Isolate

The homogeneity of the purified lectin was confirmed and established by disc electrophoresis on polyacrylamide gel. A single band was observed at pH 8.8 under non-denaturing and denaturing condition. From the calibration curve (Fig. 3) constructed, the approximate molecular weight of the lectin was calculated to be 65 kD.

The purified lectin was found to contain 10.96% total sugar by the phenol-sulfuric acid method. The sugar components was determined by High Pressure Liquid Chromatography using different monosaccharide standards for comparison. However, only one broad peak was observed

in the chromatogram of the sample which did not correspond to any of the peaks of the standard. It is possible that hydrolysis of the sample was not completed such that an oligosaccharide came out of the chromatogram instead of a monosaccharide. The identity of the peak was not established because of the unavailability of oligosaccharide standard.

The glycoprotein nature of the purified lectin was further established by determining the sugar content of the eluted fraction of the solubilized crystal protein (Fig. 4). The profile clearly presented the strong association of the carbohydrate and protein by the significantly higher total sugar content of the fractions with lectin activity.

Determination of Mitogenic Activity

Giemsa-stained preparation from cultures containing the positive control (*Phaseolus vulgaris* lectin), purified BT lectin and a negative control (no lectin) were examined morphologically under the microscope. The presence and/or abundance of transformed lymphocyte, termed as lymphoblast was noted in the positive control and in the purified BT lectin (Fig. 5). Lymphocyte and lymphoblasts were differentiated from each other in terms of the cell size, the basophilia of the cytoplasm, the structure of the nucleus and the presence of nucleoli. The characteristic feature of a lymphocyte is a relatively large nucleus surrounded by a thin layer of clear, homogeneous, sky blue cytoplasm. The nucleus is nearly spherical but on one side it has a more or less obvious indentation. In stained preparation the chromatin forms a thick layer at the nuclear envelope and several darkly staining masses in the interior.

Lymphoblast, on the other hand, is a large cell (>10 um) having a large nucleus, with heavier, coarser and denser chromatin. The nuclei very rarely number more than (2) two are sharper and nearly spherical. The narrow rim of clear blue and homogeneous cytoplasm often shows a pale crescent-like area next to the nucleus (Raphael, 1976; Ham & Cormack, 1979; Bloom & Fawcett, 1968). Lymphocytes develop into cells specialized to provide immunological defenses for the body. There are 2 kinds of lymphocytes, the B- & T- type. The thymus or T lymphocyte is directly related with cellular immunity, while the B or bone marrow lymphocyte is responsible for humoral or antibody immunity (Nowell, 1960).

Results of this experiment suggest that BT lectin has mitogenic activity. Of the approximately 300 cells counted, about 43% of the lymphocytes were found to be transformed after 72 hours. For confirmation of the mitogenic activity, radioactive assay using [^{3}H] thymidine is recommended.

Bioassay for Toxicity

For the toxicity assay three-day old (second instar) larvae of *A. albopictus* were exposed to two concentrations of the crude extract, the purified lectin and the purified non-lectin protein at a maximum of 72 hour exposure.

Results showed that a 1 hr exposure of the larvae in three solutions at 0.50 mg/ul concentration, resulted to 100 percent mortality (Table 4). At 10 times dilution of the concentration a relatively lower mortality rate was observed for the crude extract while no mortality was observed for the lectin and the non-lectin fraction after 1 hr of exposure. A 100% mortality rate was observed for the crude extract only after 24 hr. of exposure and it needs 72 hr. to kill all the larvae for the non-lectin protein. For the purified lectin, only a 29% mortality rate was observed after 72 hours.

It is interesting to note that the crude extract gave 100% mortality after 1 hr of exposure which shows that the protein extract contain the toxin. A dilution of the extract lowers the percent

mortality and lengthens the life of the larvae. When the purified lectin was used, a 100% mortality was also obtained at a high protein concentration. However, when the concentration was decreased, a 17% mortality was obtained only after 24 hr.. A high salt concentration was demonstrated to contribute to the toxicity of the protein.

It will also be noted that the crude extract is the most toxic of the three samples. Toxicity seems to be lowered when the lectin was used alone. Likewise, toxicity also decrease when the nonlectin protein was used alone. From these preliminary results, it would seem that the non-protein fraction may contain the toxin because of a relatively higher mortality rate compared to the lectin fraction. However, it is very clear that the lectin fraction also exhibits toxicity. Although the results of the assay is quite preliminary, there seems to be an indication that the lectin might be involved in exerting toxicity. Further studies will be done along this line to determine the role of lectin in toxicity.

Most of the insecticidal activity of *B. thuringiensis* had been attributed to the delta-endotoxin. However, there are some reports that other factors produced by *B. thuringiensis* might be significant in the insecticidal action to insects. Stahley et.al. (1989) reported that beta exotoxin is widely toxic to a variety of insects, both larvae and adults. This exotoxin is a heat stable nucleotide analog that is an inhibitor of insect, mammalian and bacterial RNA polymerases. On the other hand, the alpha-exotoxin causes breakdown of essential phospholipids in the insect tissue (Falcon, 1971). The protein which is active against mosquitoes has not been well established. Some reports demonstrated the 28 KD protein or its cleavage product of 25 KD to be the mosquitocidal protein while others attributed the toxicity to the 65 KD polypeptide, while some other authors (Calabrese et.al., 1980; Glatron et.al., 1972; Huber et.al., 1981) associated the toxicity to the 130 KD polypeptide.

In this study, a 65 KD lectin was found to exhibit a weak mosquitocidal activity at a lower concentration of the protein. This could possibly be due to the report (Thiery, 1987) that three main polypeptides of MW 28 KD, 65 KD and 130 KD must co-exist to exhibit full toxicity. The other polypeptides which could be essential for activity may not be lectin protein

SUMMARY AND CONCLUSION

A lectin has been isolated and purified from the spores/crystals of *B. thuringiensis* subsp. *morrisoni* (Serotype H 8a:8b), PG-14. Partial purification was done by solubilization at alkaline pH 9.5 in the presence of dithiothreitol (reducing agent) and $(NH_4)_2SO_4$ fractionation. Further purification was accomplished by gel chromatography on Sephadex G-200. The homogeneity of the purified lectin was determined by polyacrylamide gel electrophoresis under non-denaturing conditions.

The purified lectin was found to be non-specific because it agglutinated all types of human blood (A,B and O) and animal blood (calf and goat). The positive effect of trypsin treatment on agglutination was shown by an increase in the titer values. However, inhibitory effect of different sugars were not observed even at high sugar concentration (1000 mM). Confirmation of the purity of the lectin by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed that it contained only one single polypeptide band of molecular weight approximately 65 kD. It was found to contain 10.96% total sugar by the phenol-sulfuric acid method. The presence of carbohydrate moiety was established by the results of high pressure liquid chromatography (HPLC). A peak observed probably corresponds to an oligosaccharide based from its retention time, although the exact identity of the sugar was not established due to lack of sugar standards.

The purified lectin showed mitogenic property against human peripheral lymphocytes (type O). Morphological studies indicated that after 72 hours about 43% of the cell population has been transformed by the purified lectin. A relatively weak mosquitocidal activity was observed in the toxicity assay using 3-day old *Aedes albopictus* larvae. Only 29% mortality rate was observed after 72 hours exposure to lectin

RECOMMENDATIONS

The identity of the sugar composition of the purified lectin from *B. thuringiensis* subsp. morrisoni (Serotype H 8a 8b), PG-14 can be established by further modifying the hydrolysis conditions and by using other standards in the HPLC analysis. Amino acid analysis should also be done to establish the amino acid composition of the purified lectin.

More sugar standards like sugar glycosides and oligosaccharides can be used to establish the sugar specificity of the lectin. More replications on the toxicity assay is suggested to establish which fraction is responsible for larvicidal activity. Likewise, determination of the lethal concentration (LD_{50}) will help in establishing the toxicity of the purified lectin.

ACKNOWLEDGMENT

We wish to thank Dr. Leo Padua of BIOTECH for giving us the BT isolates used in the experiment. Likewise, we wish to acknowledge the support of the Institute of Chemistry, UPLB for providing funds for this work

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Table 1. Purification of lectin from B. thuringiensis subsp. morrisoni (Serotype H 8a:8b), PG-14.

STAGES OF PURIFICATION	PROTEIN CONCENTRATION (mg/mL)	TITER[1] Human Blood Type			AGGLUTINATION[2] Human Blood Type			SPECIFIC ACTIVITY[3] Human Blood Type			PURIFICATION FOLD Human Blood Type		
		A	B	O	A	B	O	A	B	O	A	B	O
I Crude Spores/ Crystals	3.24	4	4	4	0.40	0.40	0.40	1.23	1.23	1.23	-	-	-
II Solubilized Crystal Protein	0.85	2	2	2	0.21	0.21	0.21	2.35	2.35	2.35	2	2	2
III Purified Lectin	0.51	4	4	4	0.06	0.06	0.06	7.84	7.84	7.84	6	6	6

[1]Titer is defined as the reciprocal of the highest dilution with visible agglutination.

[2]Agglutination titer is defined as the least concentration of lectin to cause agglutination.

[3]Specific activity is defined as titer divided by protein concentration.

Table 2. Effect of trypsin treatment of the different blood samples on the agglutination reactions of the *B. thuringiensis subsp. morrisoni* (Serotype H 8a:8b), PG-14 lectin at different stages of purification.

STAGES OF PURIFICATION	TITER									
	Normal Red Blood Cells					Trypsinized Red Blood Cells				
	Human Blood Type			Animal Blood		Human Blood Type			Animal Blood	
	A	B	O	Goat	Calf	A	B	O	Goat	Calf
I Crude Spores/ Crystals	2	2	2	1	2	4	4	4	2	4
II Solubilized Crystal Protein	2	2	2	2	2	2	2	2	4	4
III Purified Lectin	4	4	4	+	+	8	8	8	+	+

Table 3. Hapten inhibition test on the purified lectin from *B. thuringiensis subsp. morrisoni* (Serotype H 8a:8b), PG-14.

CARBOHYDRATES	AGGLUTINATION														
	50mM*			100mM			250mM			500mM			1000mM		
	A	B	O	A	B	O	A	B	O	A	B	O	A	B	O
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Fucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Rhamnose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Xylose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Raffinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Melibiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
N-Acetylgalactosamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
N-Acetylglucosamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Methyl-alpha-D-Mannopyranoside	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

*sugar concentration

Table 4. Mosquitocidal activity of the BT lectin against 3-day old *Aedes albopictus*.

Sample No.	Protein Concentration (mg/ml)	Percent Mortality After				
		1hr.	2hr.	3hr.	24hr.	72hr.
Crude Extract	0.50	100				
	0.05	38	58	64	100	
Purified Lectin (Peak 1)	0.50	100				
	0.05	-	-	-	17	29
Non Lectin Protein (Peak 2)	0.50	100				
	0.05		-	40	60	100

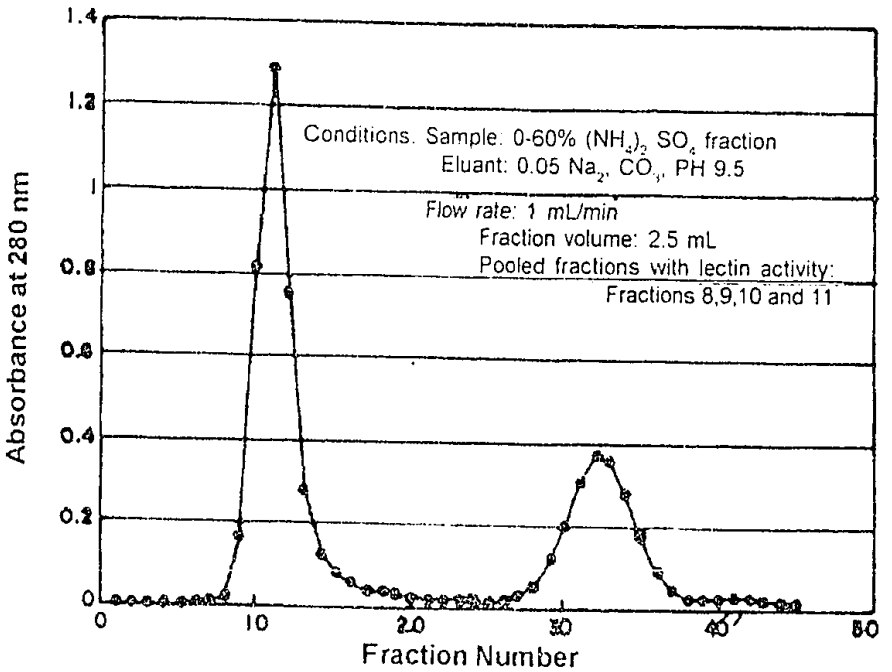

Figure 1. Gel chromatography on Sephadex-200 of the solubilized crystal protein from *B. thuringiensis* subsp. *morrisoni* (Serotype H 8a:8b), PG-14.

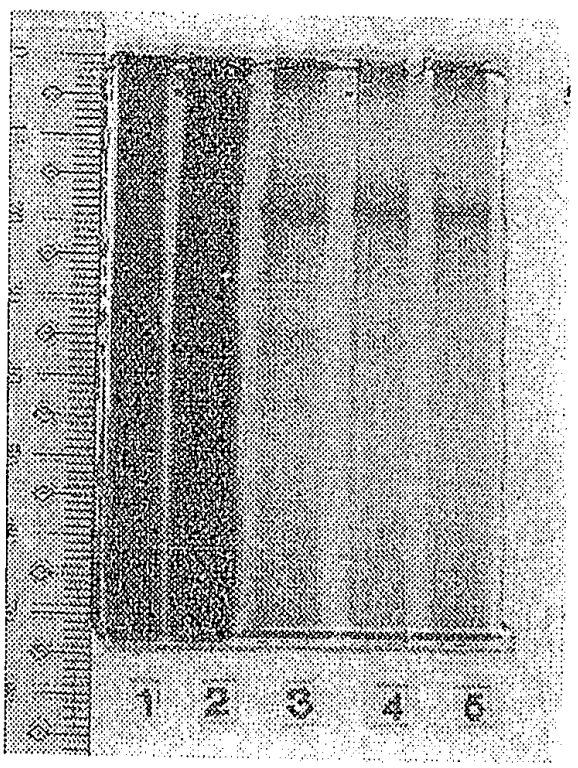

Figure 2. Polyacrylamide gel electrophoresis (PAGE) of *B. thuringiensis* subsp. *morrisoni* (Serotype H 8a:8b), PG-14 lectin at different stages of purification: (1-2) crude spores/crystals, (3-4) solubilized crystal protein and (5) purified lectin.

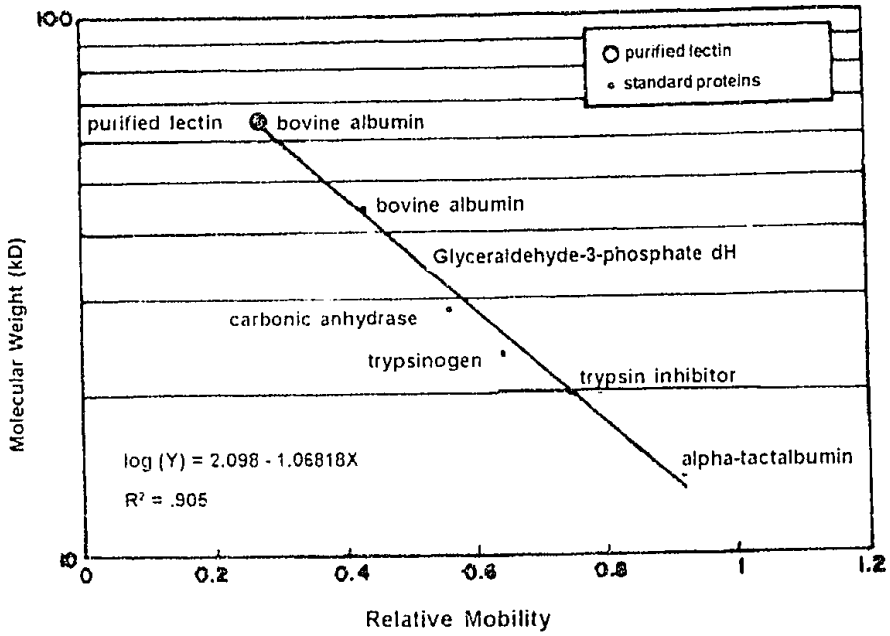

Figure 3. Calibration curve for the estimation of the molecular weight of purified *B. thuringiensis* subsp. *morrisoni* (Serotype H 8a:8b), PG-14 lectin.

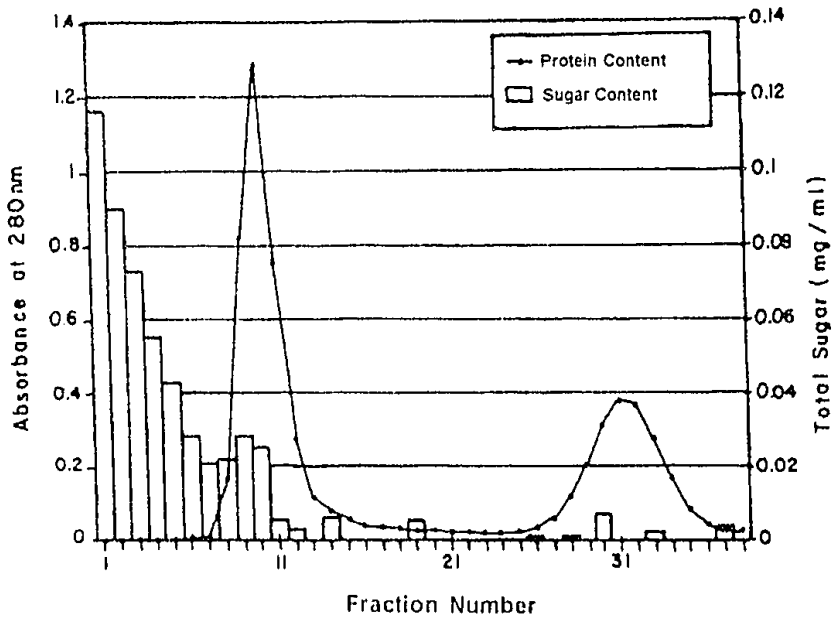

Figure 4. Total sugar and protein content of eluted fractions of solubilized crystal protein from *B. thuringiensis* subsp. *morrisoni* (Serotype H 8a:8b), PG-14 on Sephadex G-200.

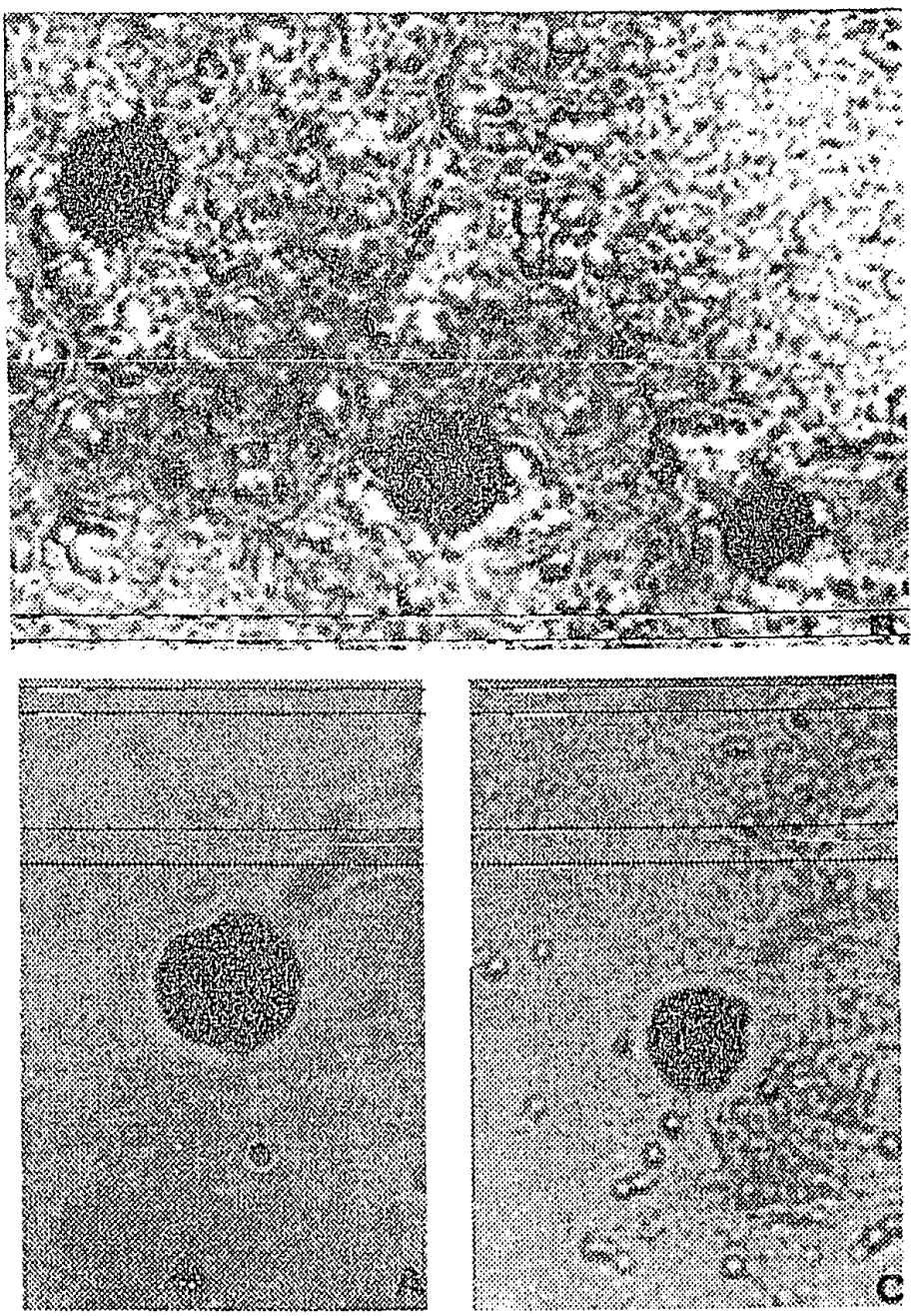

Figure 5. Photomicrographs of (A) a lymphocyte stimulated by *Phaseolus vulgaris* lectin, (B) lymphocytes stimulated by the *B. thuringiensis* subsp. *morrisoni* (Serotype H 8a:8b), PG-14 lectin and (C) a mature lymphocyte (40x).

DETERMINATION OF MIXTURES OF CARBOXYLIC ACIDS AND SELECTED INORGANIC ANIONS BY ION-EXCLUSION CHROMATOGRAPHY[1]

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ABSTRACT

Mixtures of aliphatic carboxylic acids and inorganic anions were determined by ion-exclusion chromatography. A range of sulfonic acid eluents were examined using UV absorption and conductivity detection. Of the eluents studied, sodium octanesulfonate proved to be the optimum. The aliphatic carboxylic acids were found to elute in the order of increasing carbon number. The retention of these solutes was controlled by a combination of ion-exclusion through the Donnan potential and hydrophobic interactions. Detection limits obtained were in the range of 0.71-100 ppm. Sulfonic acid eluents were found to be suitable for the determination of mixtures of aliphatic carboxylic acids and inorganic anions.

INTRODUCTION

Determination of the pollutants level in water has recently become the global concern. Water pollutants, such as mixtures of carboxylic acids and inorganic anions have been a problem in standard wastewater treatment. The presence of high levels of low molecular weight carboxylic acids in certain aqueous system (e.g.coal conversion waste waters and municipal waste leachate) could be a carbon source for microbial growth.

Quantitative methods for measurement of low molecular weight carboxylic acids exist but all have limitations. The methods involve tedious extraction and concentration procedures to increase sensitivity in liquid chromatographic determinations. Other methods such as ion-exchange (Bouyoucos,1982 and Rokushika S., Kihara K., Subosa, P.F. and Leng Wen-Xue, 1990) and reverse-phase (Manning D.L., Maskarinec M.P,1983) have been used. These approaches were also found to have problems in the extent of separation achieved. The employment of ion-exclusion techniques has overcome some of the observed difficulties.

In ion-exclusion chromatography, weak acids such as carboxylic acids, amino acids sugars, alcohols and other substances are separated on an ion-exclusion column. The separation principles involve exclusion by effect similar to the Donnan membrane equilibrium (Haddad P.R. and Jackson P.E., 1990). Other effects, such as size exclusion (Waki and Tokunaga Y., 1982; Hicks K.B., Lim P.C. and Haas M.J., 1985) and hydrophobic adsorption (Haddad, op.cit.) also govern retention.

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[1]Extracted and condensed from one of the research works for Master of Degree.

Early work in the determination and separation of carboxylic acids was performed using deionized water. Using this type of eluent, however, yielded broad and unsymmetrical peaks for solutes that are retained. The possible use of other eluents were then investigated, for instance, dilute solutions of strong and weak acids.

Of the eluents currently used in ion-exclusion chromatography, sulfonic acids have not been exploited to any significant extent. In this study, therefore, sulfonic acids as eluents are used. They are fully ionized in aqueous solutions over a wide pH range thereby eliminating system peaks. Selectivity effects arising from the variation of the eluent concentration of a range of sulfonic acids are investigated. These selectivity effects are then applied in the separation and determination of mixtures of carboxylic acids (C_1-C_8) and inorganic anions using ion-exclusion chromatography.

MATERIALS AND METHODS

The ion chromatograph used consisted of a Waters Assoc.(Milford, M.A., USA) Model MU6 K injector, Model M-481-A2 variable wavelength detector, Model M430 conductivity detector and Model M-730 data module. Column used was BioRad Aminex HPX-87 H ion-exclusion, 300x7.8 mm ID stainless steel column packed with sulfonated divinyl benzene-styrene copolymer based resin, 9μm particle size, 8% cross-linking (BioRad, Richmond, CA, USA).

All reagents used were of the highest available purity. Standard solutions of the carboxylic acids(C_1-C_8) and inorganic anions (F^-, PO_4^{-}, NO_2^-, Cl^-, Br^-, NO_3^-, SO_4^{2-}) were prepared by dissolving accurately weighed amounts of the pure acids and salts, respectively, in water purified on a Millipore Milli-water purification system and were injected directly onto the chromatograph using a micro syringe. Eluents were prepared by dissolving the desired amount in doubly distilled water. The eluents were filtered through a 0.45μm Millipore filter and degassed in an ultrasonic bath before use.

RESULTS AND DISCUSSION

Effect of the Acid Dissociation Constant (pKa)

The effect of solute charge on retention was examined for mixtures of low molecular weight aliphatic carboxylic acids or their salts and inorganic anions using various sulfonic acids as eluents. Under the chromatographic conditions used, the degree of ionization of the solutes was determined by the acid dissociation constant (pKa) of the solutes. The higher the pKa, the less ionized is the acid and the less it is excluded from the pores of the column. Plots of the retention volume *versus* pKa are given in Figures 1-6. It can be seen that fully dissociated solutes such as inorganic anions are completely excluded from the stationary phase due to repulsion by the fixed negative charge on the ion-exclusion column stationary phase. The retention volume of the eluted inorganic anions was found to be 7ml under all conditions. This volume corresponds to the column void volume, V_o. Neutral species, such as acetonitrile, are eluted at an approximate retention volume of 10.0ml (V_o+V_i). Weak acids, such as sodium formate, sodium acetate and propionic acid, are slightly dissociated and are only slightly repelled by the ion-exclusion column. They are eluted at retention volume between 7.0ml and 10ml in all eluents used except for naphthalenesulfonic acid where propionic acid is eluted outside this range. These acids are retained because they are totally uncharged and can move into the pores of the stationary phase.

The retention behavior observed is in accordance to the Donnan exclusion effect. Generally, retention times of homologous series of carboxylic acids increase as their pKa increases.

Distribution coefficient of the solute was calculated according to the Donnan exclusion equation and the measured values are shown in Table 1. Inspection of the data show that there is a strong correlation between pKa and retention volume for formic, acetic and propionic acids. The increase in distribution coefficient for the first members of the series is due to the large dissociation constant of these acids and their decreased exclusion from the fixed ions of the resin. For the remaining of the solutes, the values of D_A exceeds the theoretical value of 1.0 suggesting that hydrophobic adsorption affects the retention since the distribution coefficient increases with the chain length of the solute. The hydrophobic adsorption effect is shown in Figure 7. It can be seen that as the chain length of the carboxylic acids increases, the retention also increases.

Effect of Organic Modifiers

Generally, addition of organic modifiers, such as methanol and acetonitrile reduce the retention times of solutes. It may then be possible to manipulate the analysis time by adding organic modifiers to the eluent. In this study, only acetonitrile was used as organic modifier because it was readily available. Acetonitrile was added to all sulfonic acid eluents employed at concentrations ranging from 0.5 to 15mM. However, only the concentration that gave the best result for each eluent used was documented to avoid a voluminous documentation of data.

The effect of acetonitrile to various sulfonic acid eluents is shown in Figures 8-13. It can be seen that low molecular weight solutes (C_1-C_3) showed little change in retention with increasing acetonitrile, whereas high molecular weight solutes (C_4-C_8) showed decreased retention. The effect of the addition of acetonitrile is best illustrated in Figure 10 with octanesulfonic acids as eluent. It shows that caprylic acid (C_8) gave the greatest change in retention volume with the addition of acetonitrile.

Effect of Eluent Concentration

The goal of this study is to find a suitable eluent that can separate and determine mixtures of carboxylic acids and inorganic anions. The eluent should be sufficiently acidic to suppress the ionization of organic acids in order to give sharp chromatographic peaks. Various concentrations of different types of sulfonic acids ranging from 0.5 to 15mM were then investigated and compared using ion-exclusion chromatography. However, only concentrations that gave the best result for each eluent was recorded.

Figures 14-19 show the separation and the subsequent determination of mixtures of carboxylic acids and inorganic anions at varying eluent concentrations. There was almost no change in the retention times of C_1-C_3 acids when the concentration was varied for all of the sulfonic acids eluents investigated. However, the retention time, of the C_8 acid was observed to decrease very slightly as the concentrations of methane-, ethane-, camphor- and naphthalenesulfonic acids were decreased. Heptanoic acid and the sodium salt of caprylic acid have very long retention times and could not be detected except when methane- and octanesulfonic acids were used. In these cases, heptanoic acid was eluted at a retention time of 54.4 and 55.8 minutes, respectively. The observed retention behavior of C_1-C_3 acids can be attributed to the fact that over the range of eluent pH values used, there was little change in the degree of ionization of the solutes tested. However, when the pH of the eluent is in the vicinity of the pKa of the solute acid, more pronounced effects can be expected. The effect of pH on the retention times of solutes was shown by Kihara et al.(1987) using 1mM disodium hydrogen

phosphate.

Effect of Sulfonic Acid Eluents on Detection

Chromatograms obtained in this study showed that spectrophotometric detection proved to be a more sensitive method than conductivity detection when using methane-, ethane-, octane- and camphorsulfonic acids as eluents. This is illustrated in Figures 20-23 and is attributed to the relatively low molar absorptivities and high background conductances of the eluents under the chromatographic conditions used. Excellent separation and detection of the six carboxylic acids occurred. Completely ionized solutes (eg. NO_2^-, NO_3^-, Cl^-, Br^-, SO_4^{2-}) were eluted together with the void volume due to their ionic characteristics. Weakly ionized solute such as fluoride was found to form a shoulder peak when methane- and ethanesulfonic acids were used as eluent. Similar observation was obtained for phosphate with a camphorsulfonic acid eluent. Fluoride was also found to have considerable retention with naphthalene- and toluenesulfonic acid eluents. The observed retention of these solutes can be explained by the possible formation of weak acids, HF^- and HPO_4^{2-} during the separation process. The pKa of HF^- (2.17) and HPO_4^{3-}(3.2) are high enough to cause considerable retention. The presence of these acids, however, did not interfere in the separation process since they were eluted before the carboxylic acids.

Solutes which absorb poorly in the UV region but gave good separation with conductivity detection were highlighted for an eluent containing naphthalene- and toluenesulfonic acids. Figures 24-25 show a good separation of such solutes but detectability of the C_2-C_5 acids were poor. Reasonable detection, however, can be obtained by using a more sensitive setting and employing a higher concentration of solute mixture.

The chromatogram shown in Figure 22 reveals that both UV and conductivity detection can be employed for the determination of aliphatic carboxylic acids using octanesulfonic acid as eluent. It is, however, preferable to use UV detection due to better sensitivity. Separation and determination of solutes were achieved between C_1-C_7 acids but detection of C_8 was not possible. This shows that C_8 has a strong hydrophobic adsorption on the polymer resin. A small broad peak was detected before C_6 which could be due to an isomer of C_6. Other "dips" and tailings observable in the chromatogram could be caused by the disturbance of column equilibrium.

Table 2 lists form(s) of detection suitable for each eluent and shows the approximate detection limits for the solutes using the chromatographic conditions in Figure 20-25 and an injection volume of 25 µl. The overall results showed acceptable detection of formic, acetic, propionic, butyric and heptanoic acid. These results are better to those obtained by Widiastuti (1991) using 10 mM methanesulfonic acid with 5% acetonitrile as eluent. However, Haddad et al.(1988) has reported that detection limits in parts per-billion range can be obtained for formic, acetic, propionic and butyric acids using direct UV detection utilizing an on-line pre-concentration method.

CONCLUSION

This study has shown that separation and subsequent detection of mixtures of carboxylic acids and inorganic anions using ion exclusion chromatography was possible and governed by several factors. The most significant was the degree to which the carboxylic acids were ionized as determined by the acid dissociation constant (pKa). Low molecular weight weak acids such as formate, acetate and propionic acid showed that the retention increased with increasing pKa.

Hydrophobic adsorption is another factor that contributed to carboxylic acid retention and separation. As the alkyl chain length of these acids increased, hydrophobic adsorption effects also increased, leading to longer retention times. The retention times, however, were reduced by adding a more hydrophobic solvent such as acetonitrile. It was, therefore, possible to manipulate the analysis time by adding hydrophobic solvents (organic modifiers) to the eluent. Of the eluents examined, sodium octanesulfonate proved to be the best. Detection limits obtained for an injection volume of 20 µL were in the range of 0.71-100 ppm.

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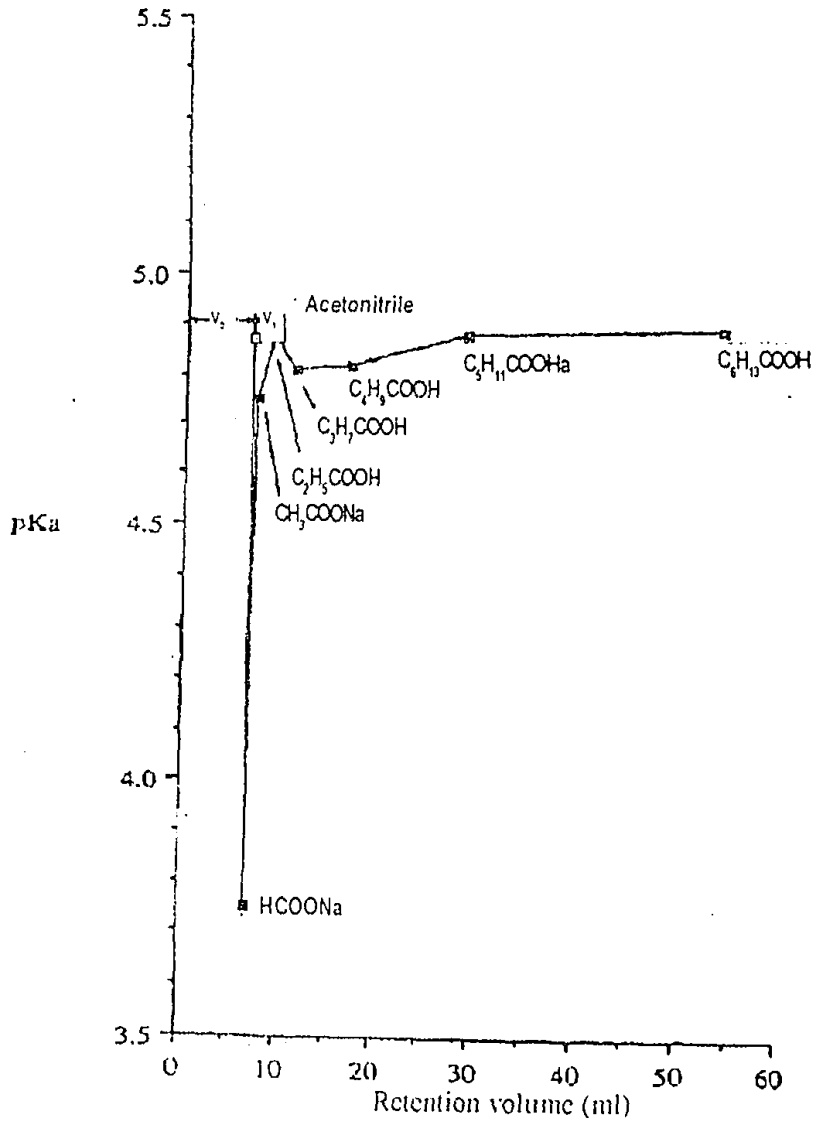

Figure 1. Relationship between retention volume and dissociation constant for carboxylic acids using methanesulfonic acid as eluent.

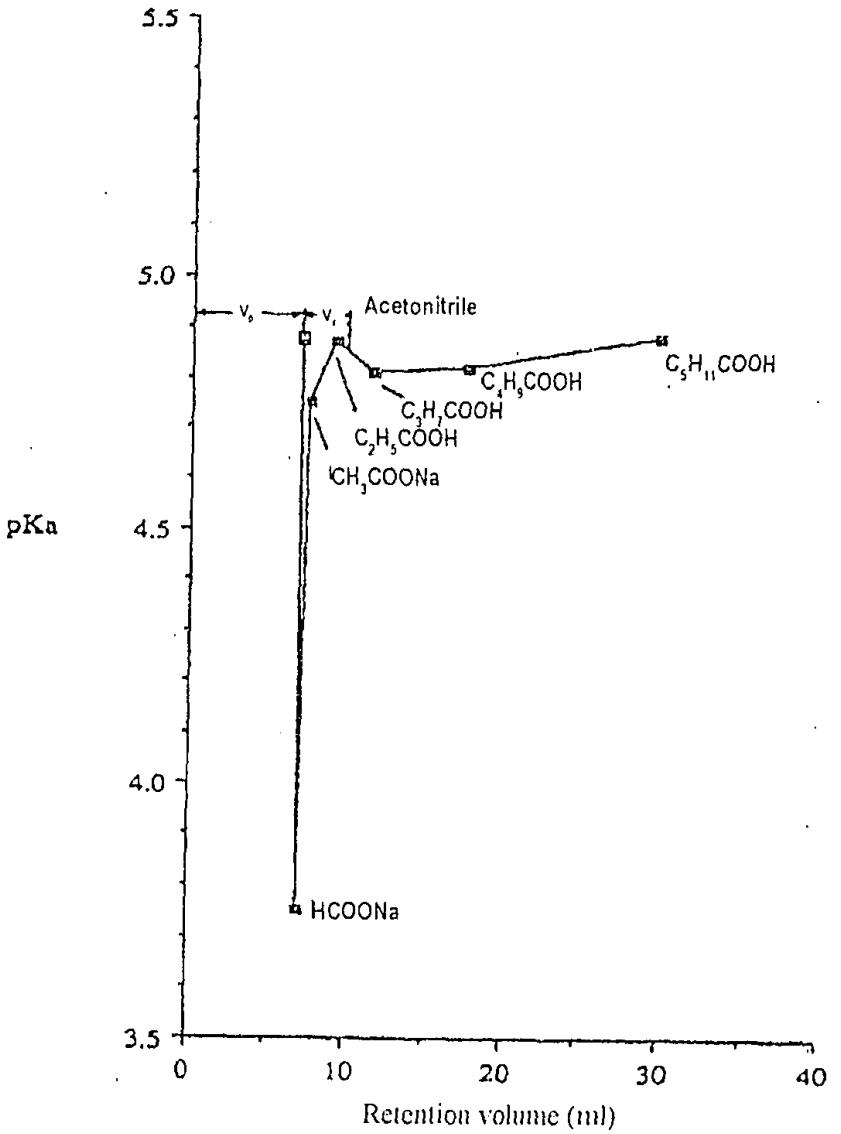

Figure 2. Relationship between retention volume and dissociation constant for carboxylic acids using ethanesulfonic acid as eluent.

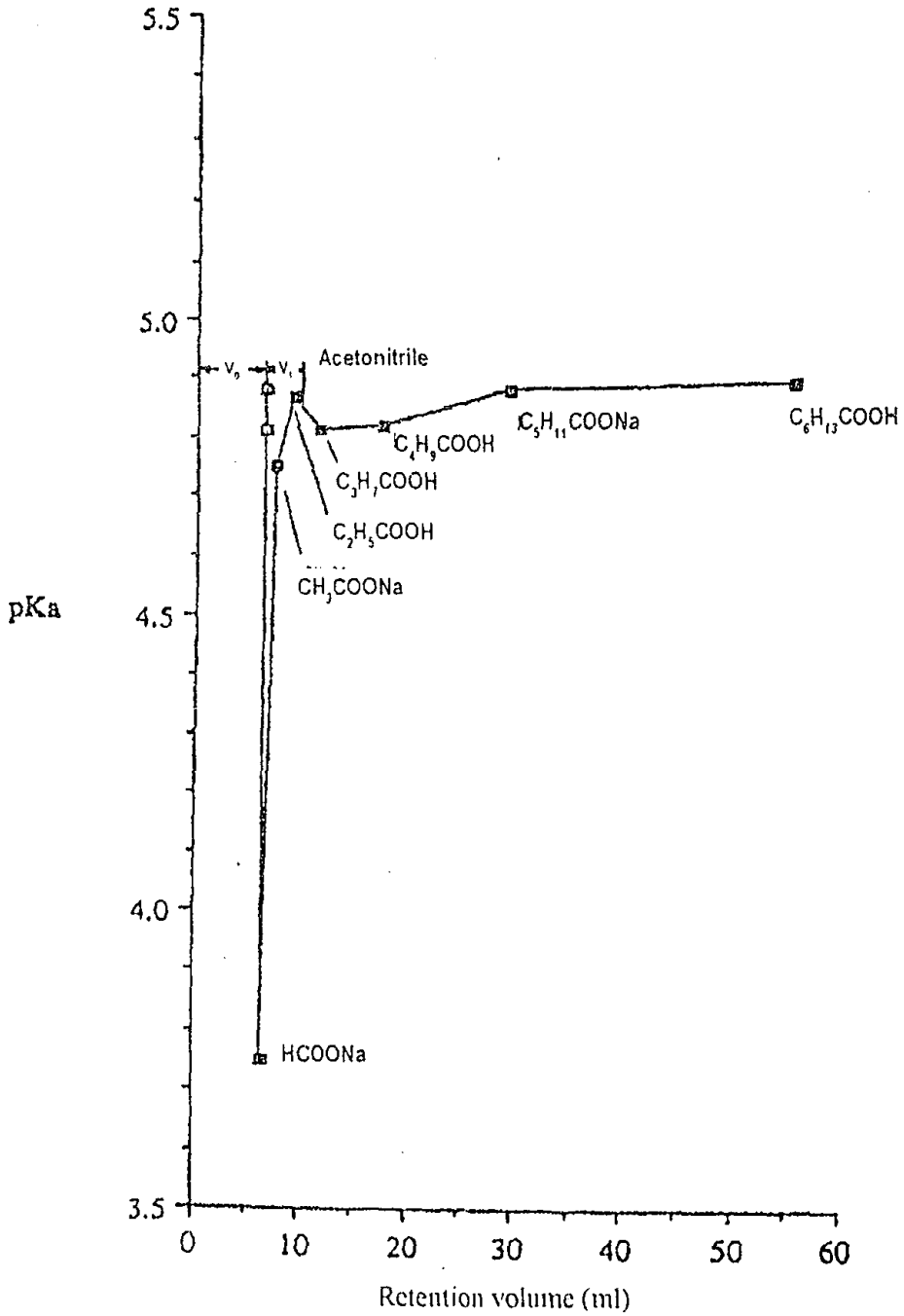

Figure 3. Relationship between retention volume and dissociation constant for carboxylic acids using sodium octanesulfonate as eluent.

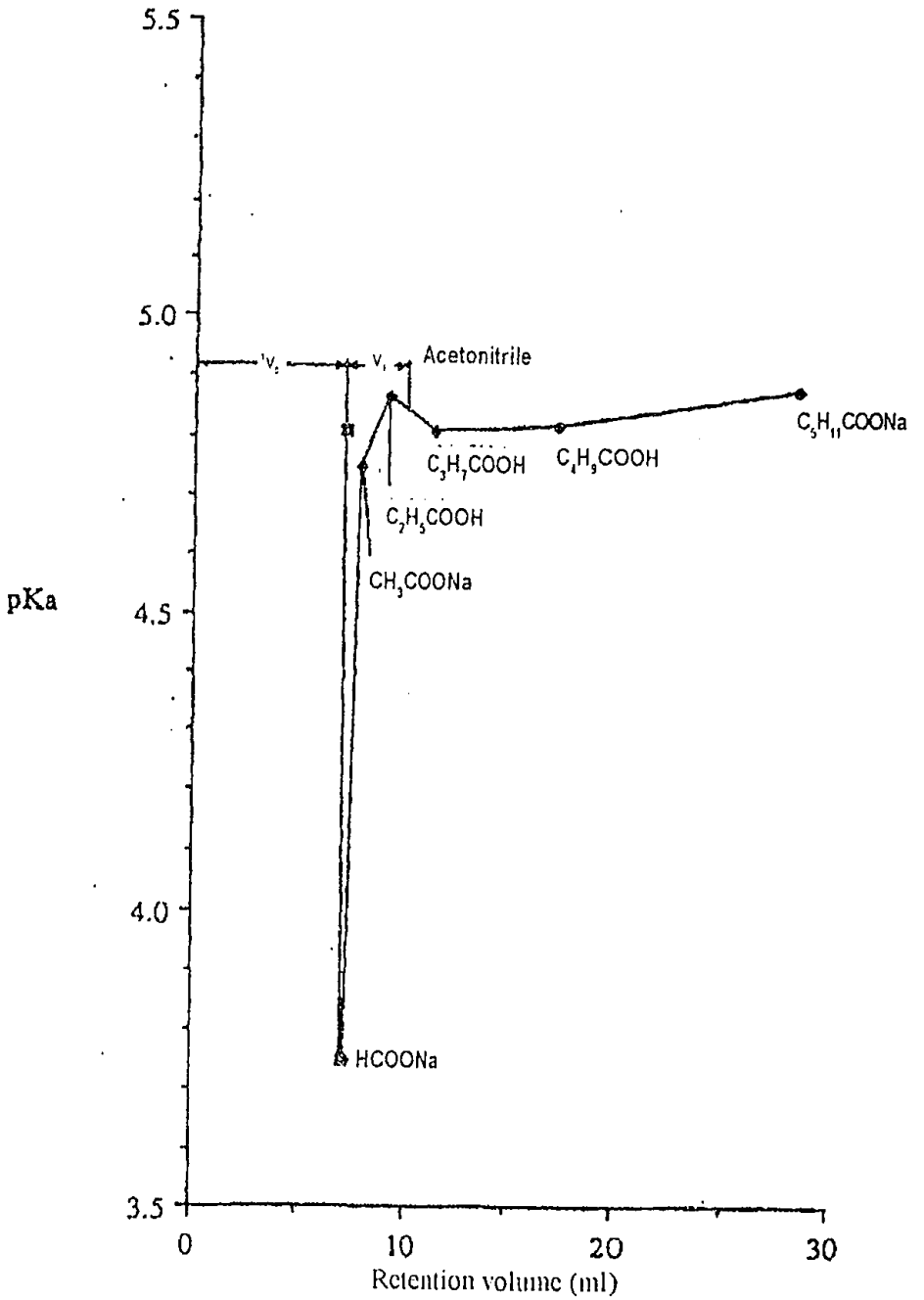

Figure 4. Relationship between retention volume and dissociation constant for carboxylic acids using camphorsulfonic acid as eluent.

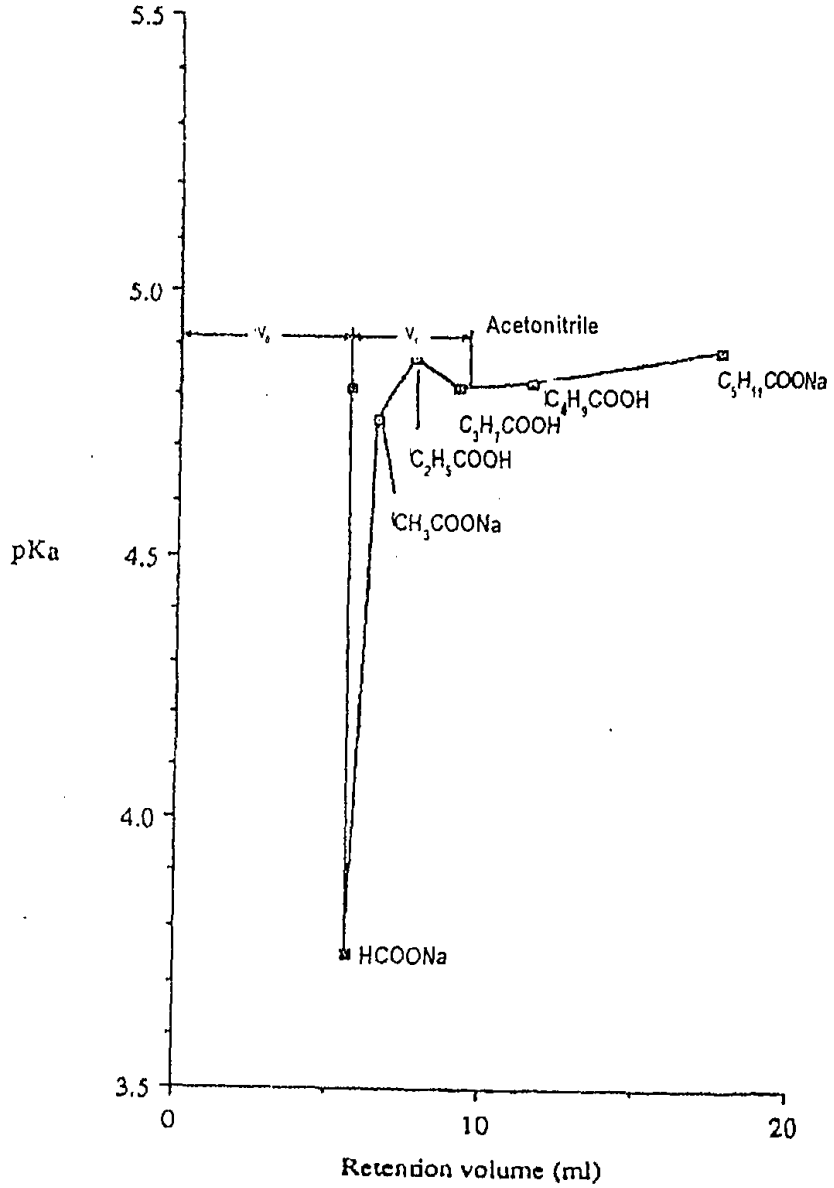

Figure 5. Relationship between retention volume and dissociation constant for carboxylic acids using toluenesulfonic acid as eluent.

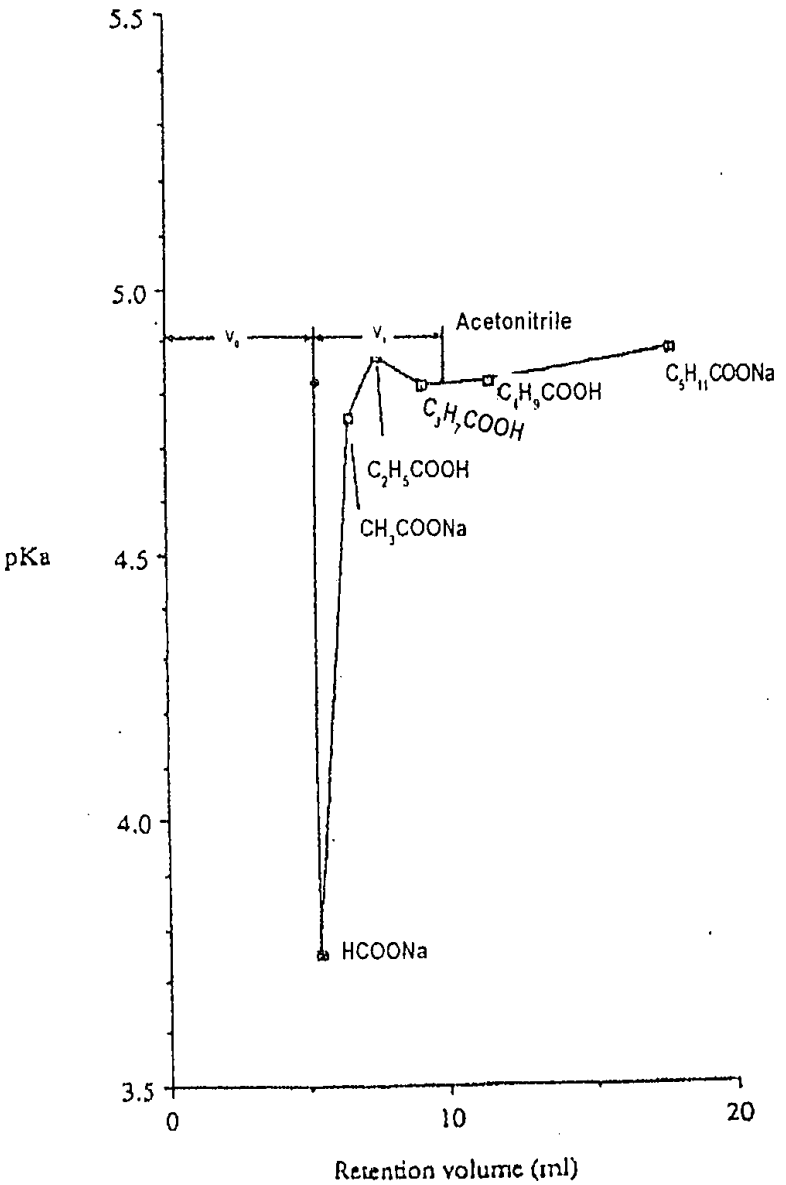

Figure 6. Relationship between retention volume and dissociation constant for carboxylic acids using naphthalenesulfonic acid as eluent.

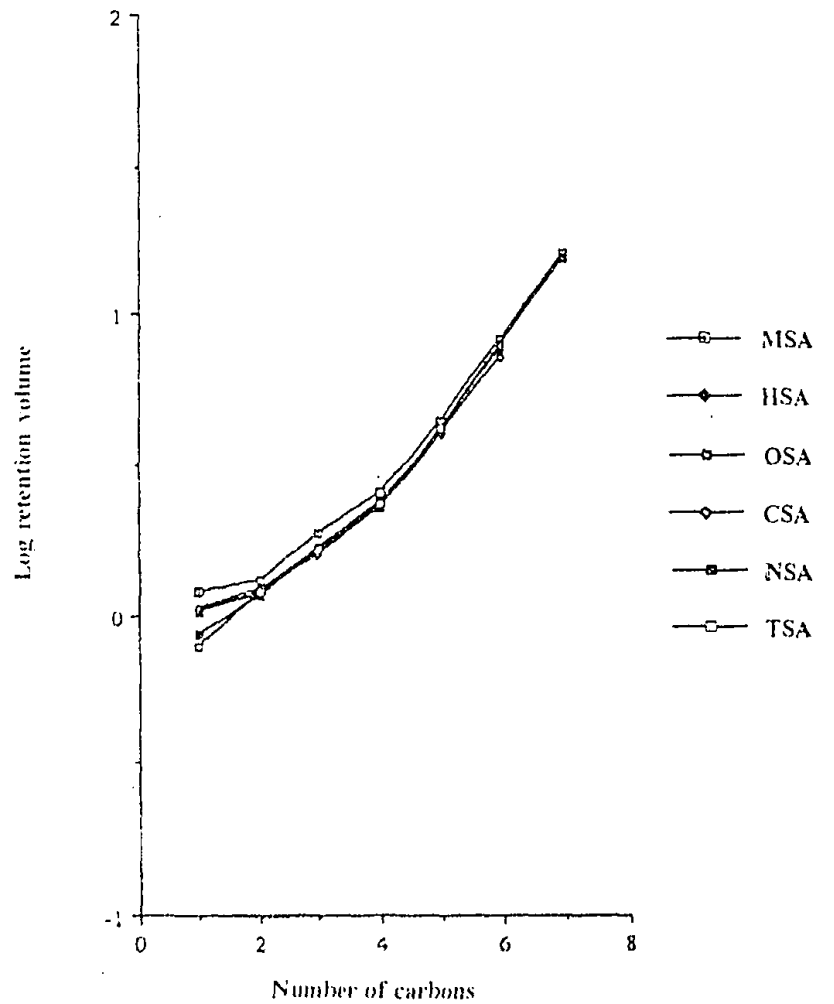

Figure 7. Relationship between the logarithm of retention volume and the carbon chain length for carboxylic acids using various sulfonic acid as eluents.

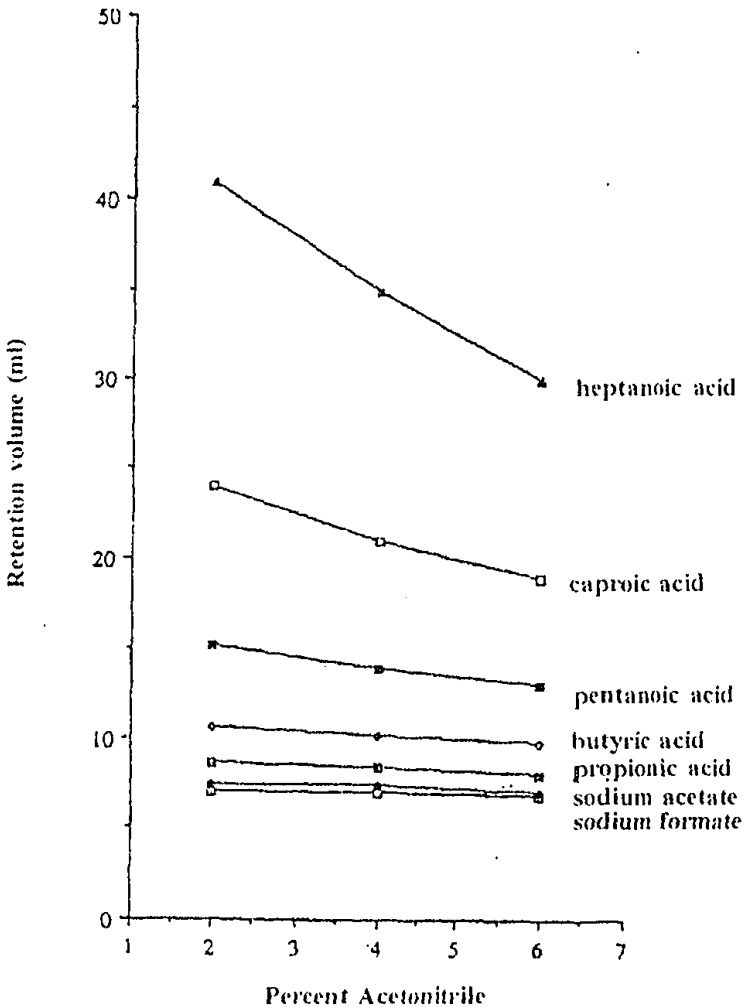

Figure 8. The effect of the addition of acetonitrile on the retention volume of carboxylic acids using 13 mM methanesulfonic acid.

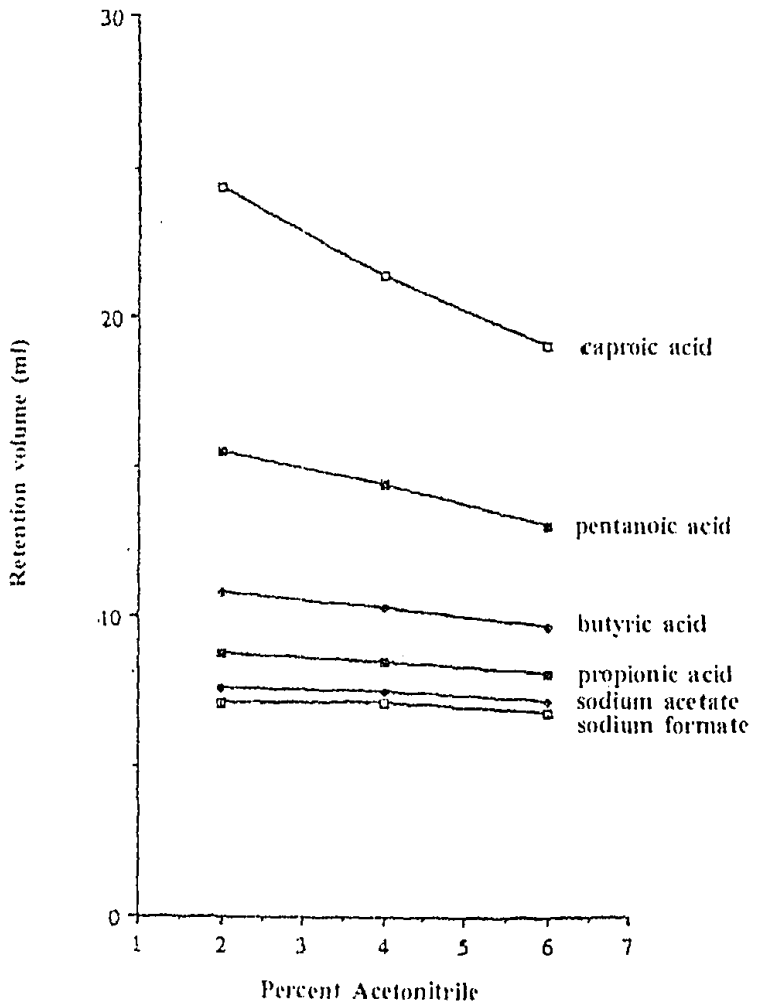

Figure 9. The effect of the addition of acetonitrile on retention volume of carboxylic acids using 13 mM ethanesulfonic acid.

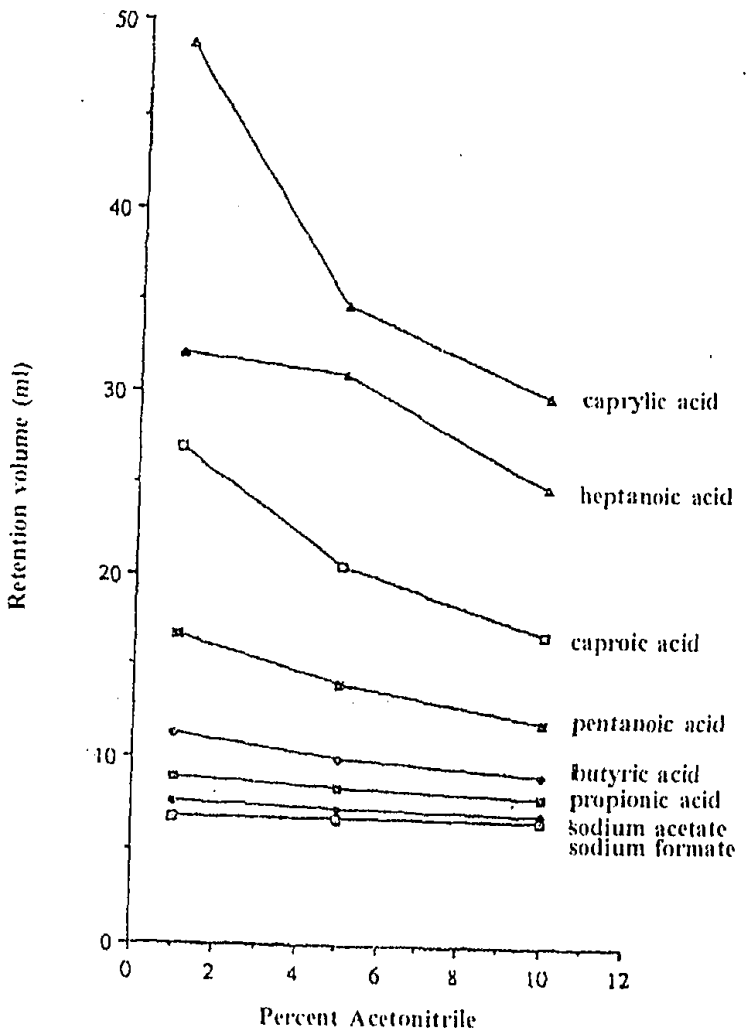

Figure 10. The effect of the addition of acetonitrile on the retention volume of carboxylic acids using 1mM octanesulfonic acid.

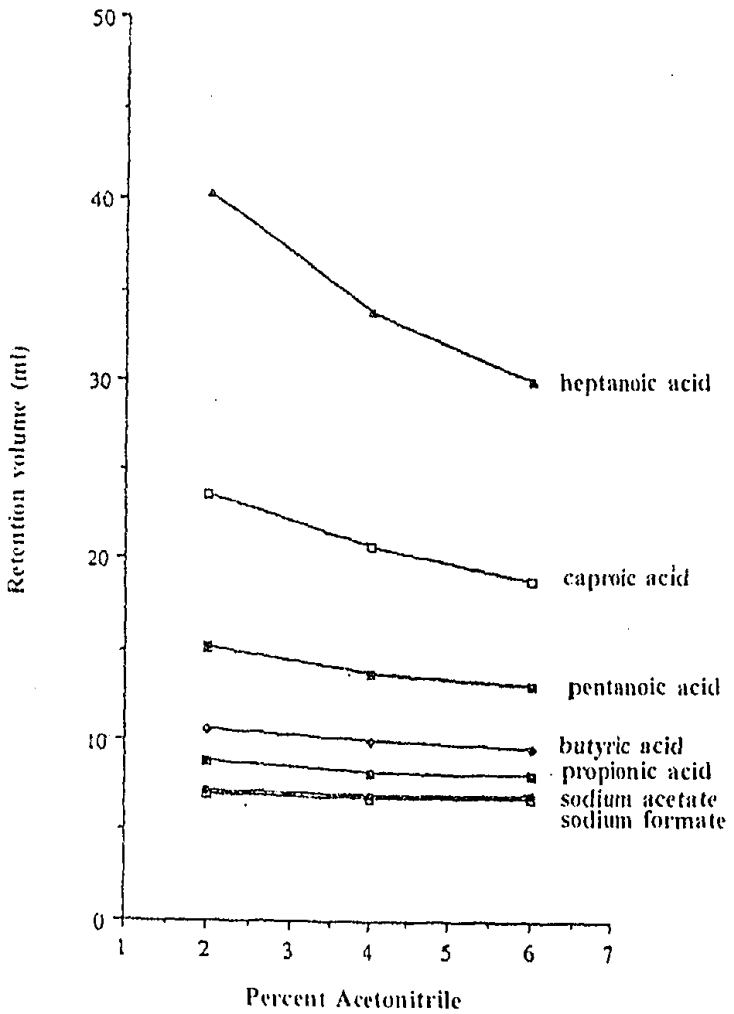

Figure 11. The effect of the addition of acetonitrile on retention volume of carboxylic acids using 10mM camphorsulfonic acid.

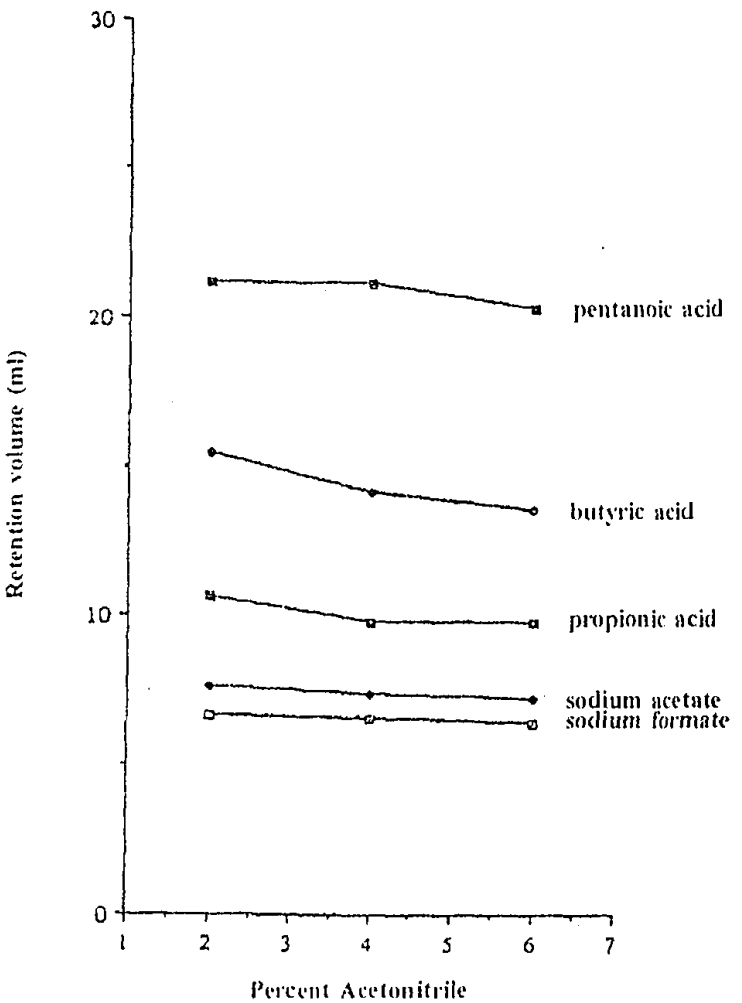

Figure 12. The effect of the addition of acetonitrile on the retention volume of carboxylic acids using 1mM naphthalenesulfonic acid with conductivity detection.

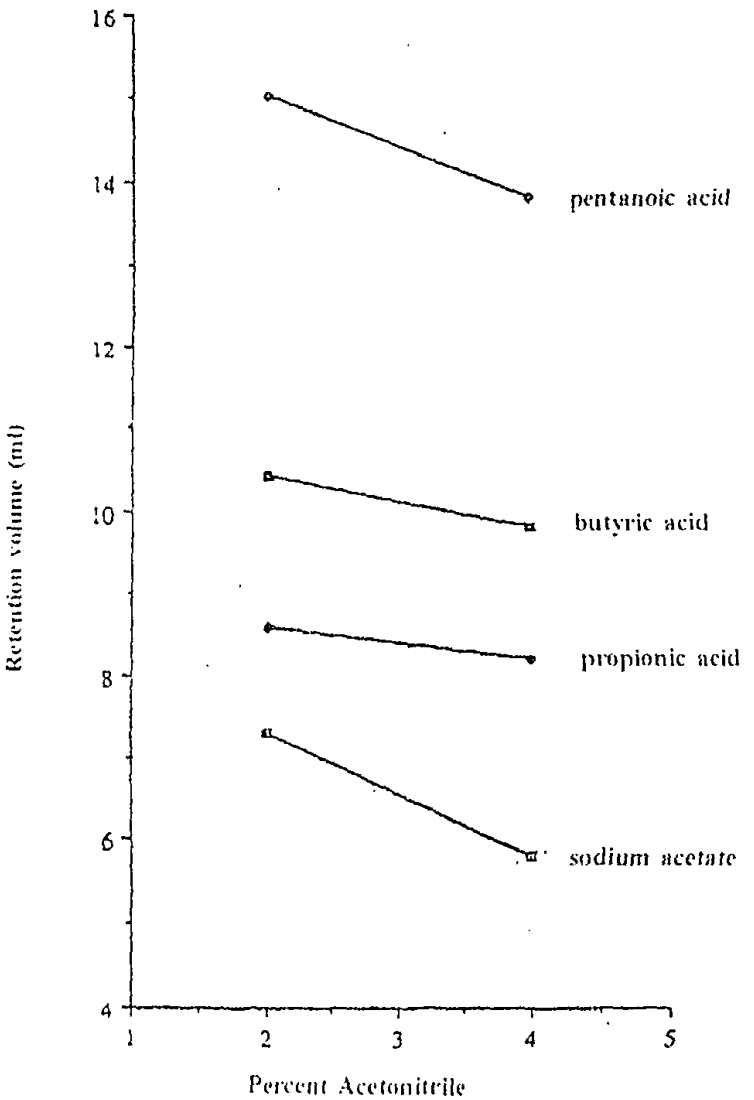

Figure 13. The effect of the addition of acetonitrile on the retention volume of carboxylic acids using 0.5mM toluenesulfonic acid with conductivity detection.

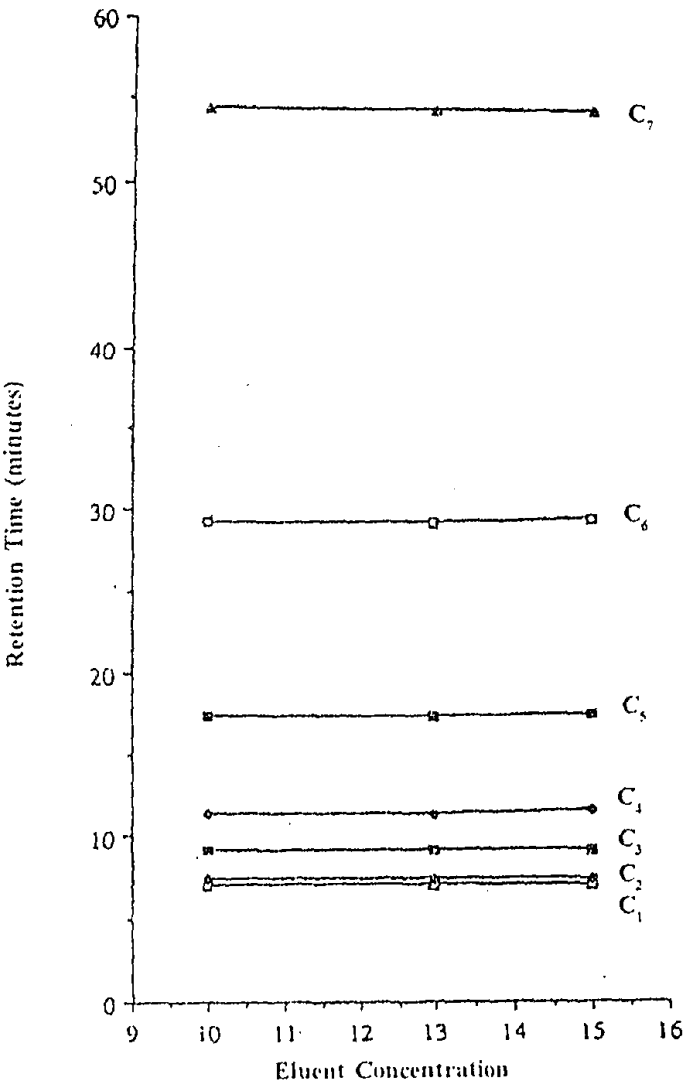

Figure 14. The effect of eluent concentration on the retention time of carboxylic acids using methanesulfonic acid eluent.

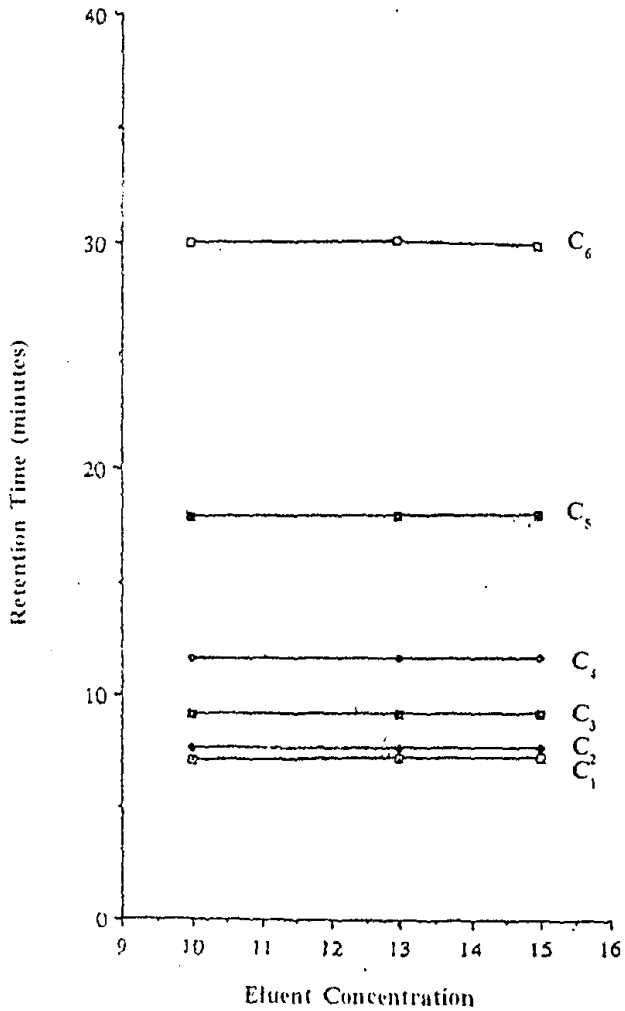

Figure 15. The effect of eluent concentration on the retention time of carboxylic acids using ethanesulfonic acid eluent.

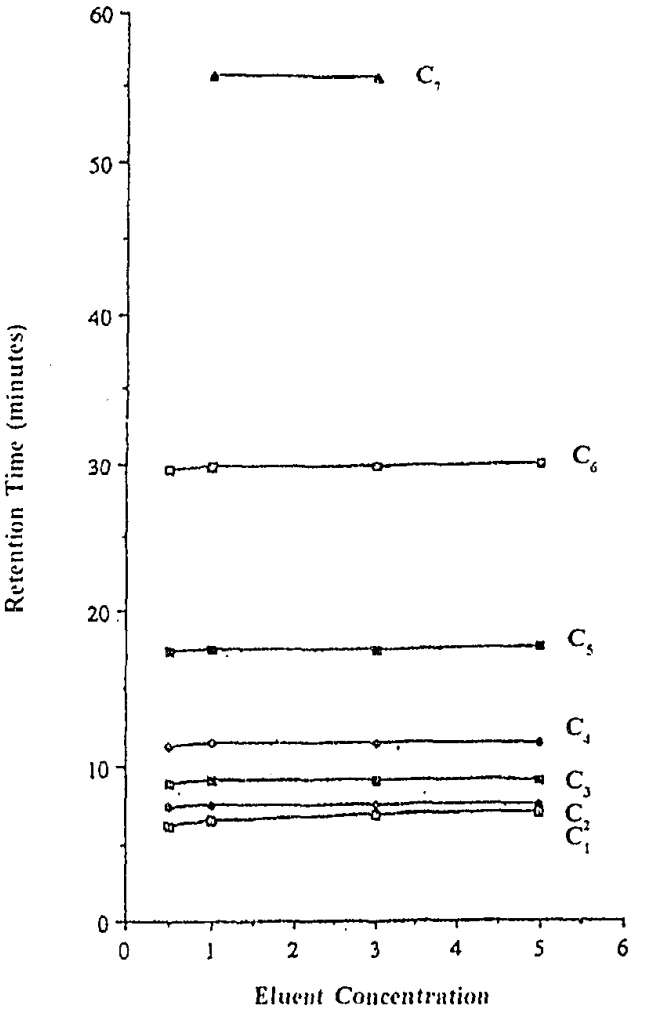

Figure 16. The effect of eluent concentration on the retention time of carboxylic acids using octanesulfonic acid eluent.

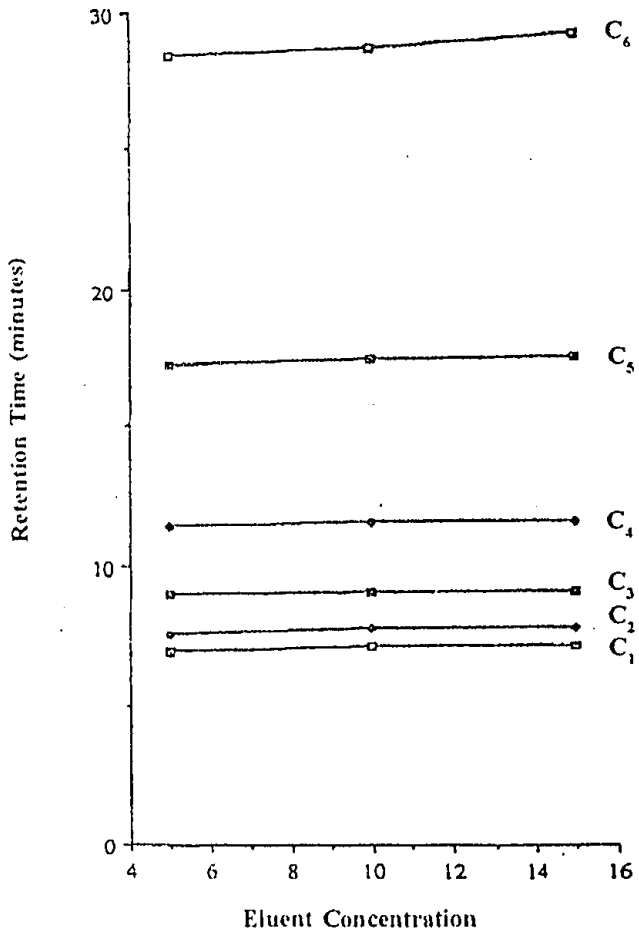

Figure 17. The effect of eluent concentration on the retention time of carboxylic acids using camphorsulfonic acid eluent.

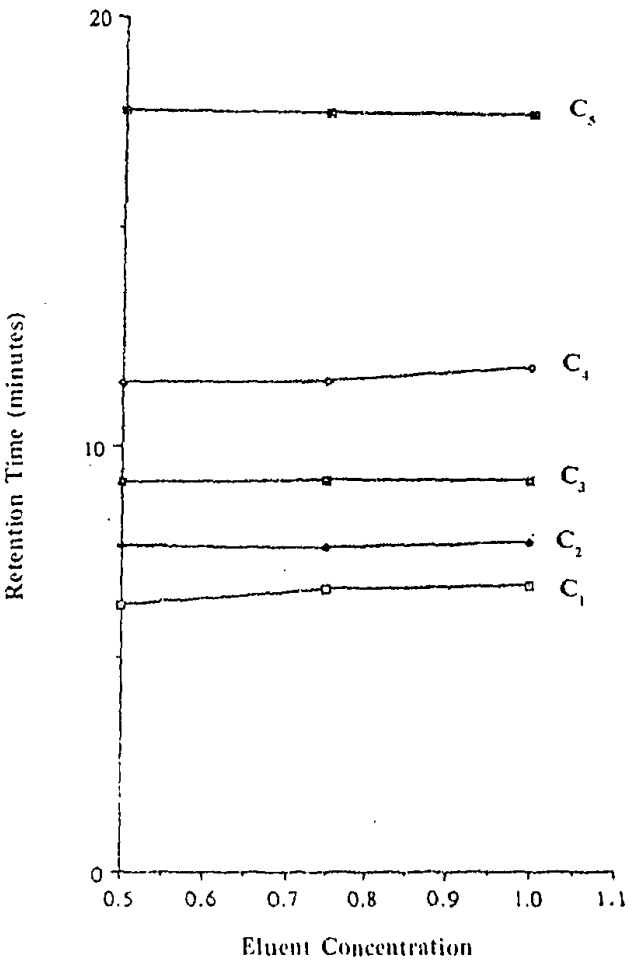

Figure 18. The effect of eluent concentration on the retention time of carboxylic acids using naphthalenesulfonic acid eluent.

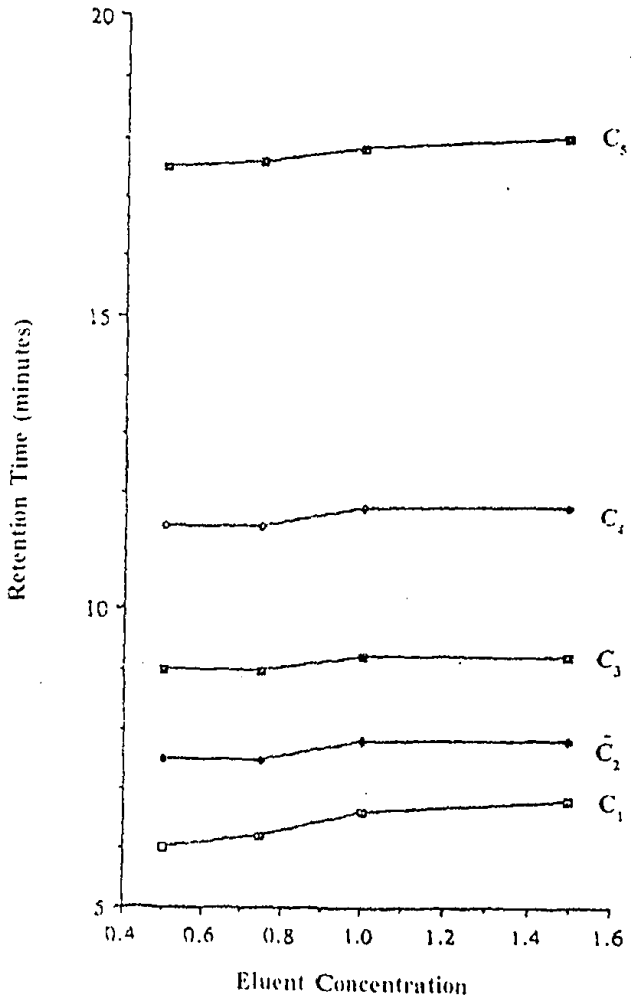

Figure 19. The effect of eluent concentration on the retention time of carboxylic acids using toluenesulfonic acid eluent.

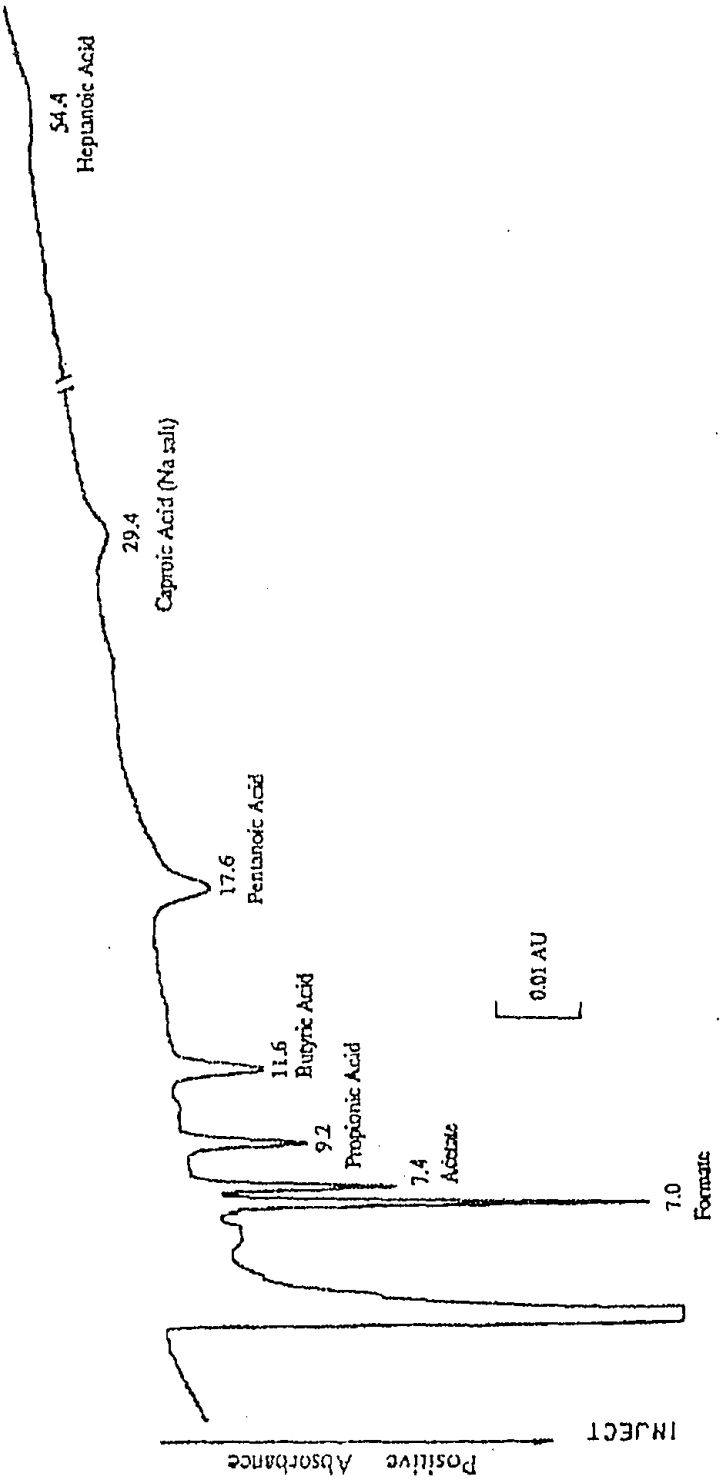

Figure 20. Chromatogram obtained with 15 mM methanesulfonic acid as eluent by direct spectrophotometric detection. *Sample:* 20 µL of a solution of 50 ppm each of the inorganic anions and aliphatic carboxylic acids. Detection wavelength 213 nm. Retention times in minutes.

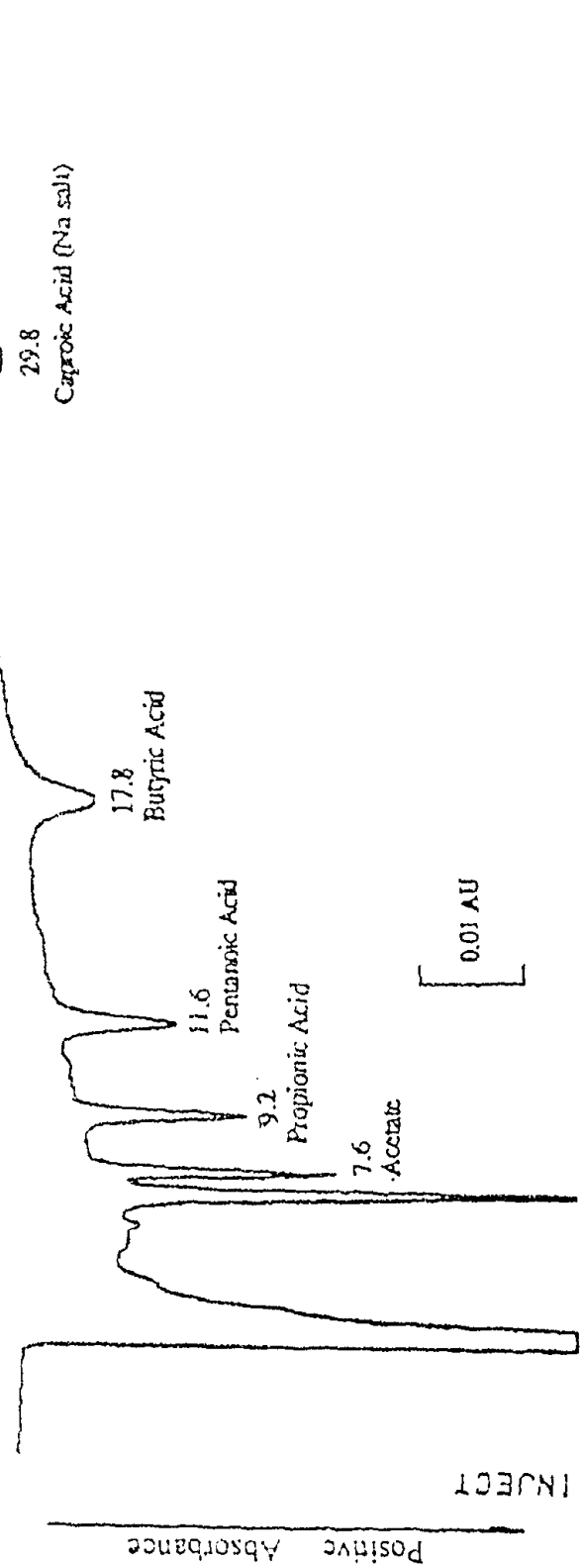

Figure 21. Chromatogram obtained with 15 mM ethanesulfonic acid as eluent by direct spectrophotometric detection. Detection wavelength 213 nm. Other conditions as in Figure 20.

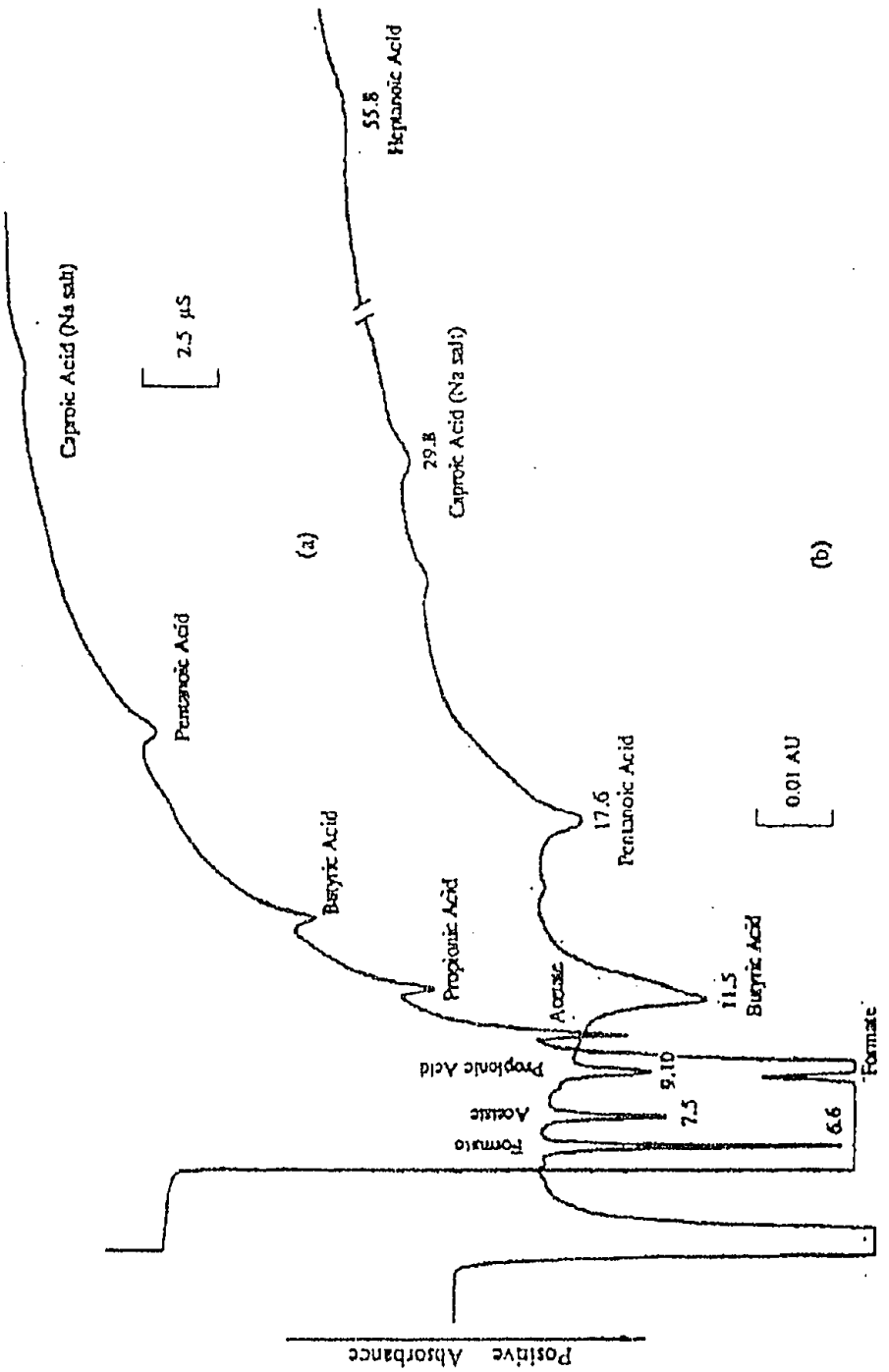

Figure 22. Chromatogram obtained with 1 mM octanesulfonic acid as eluent by use of (a) conductivity (b) direct spectrophotometric detection. Detection wavelength 220 nm. Other conditions as in Figure 20.

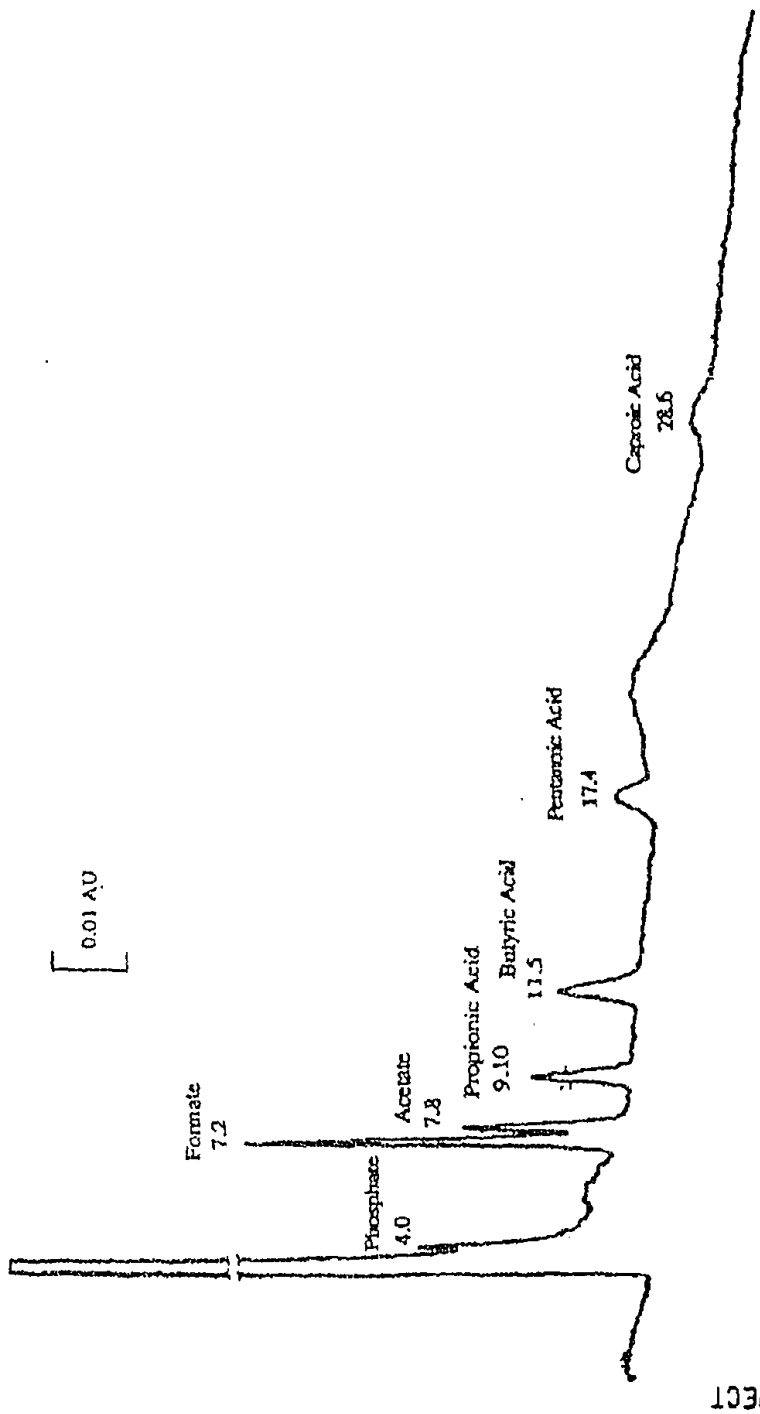

Figure 23. Chromatogram obtained with 10 mM camphorsulfonic acid as eluent by direct spectrophotometric detection. Detection wavelength 213 nm. Other conditions as in Figure 20.

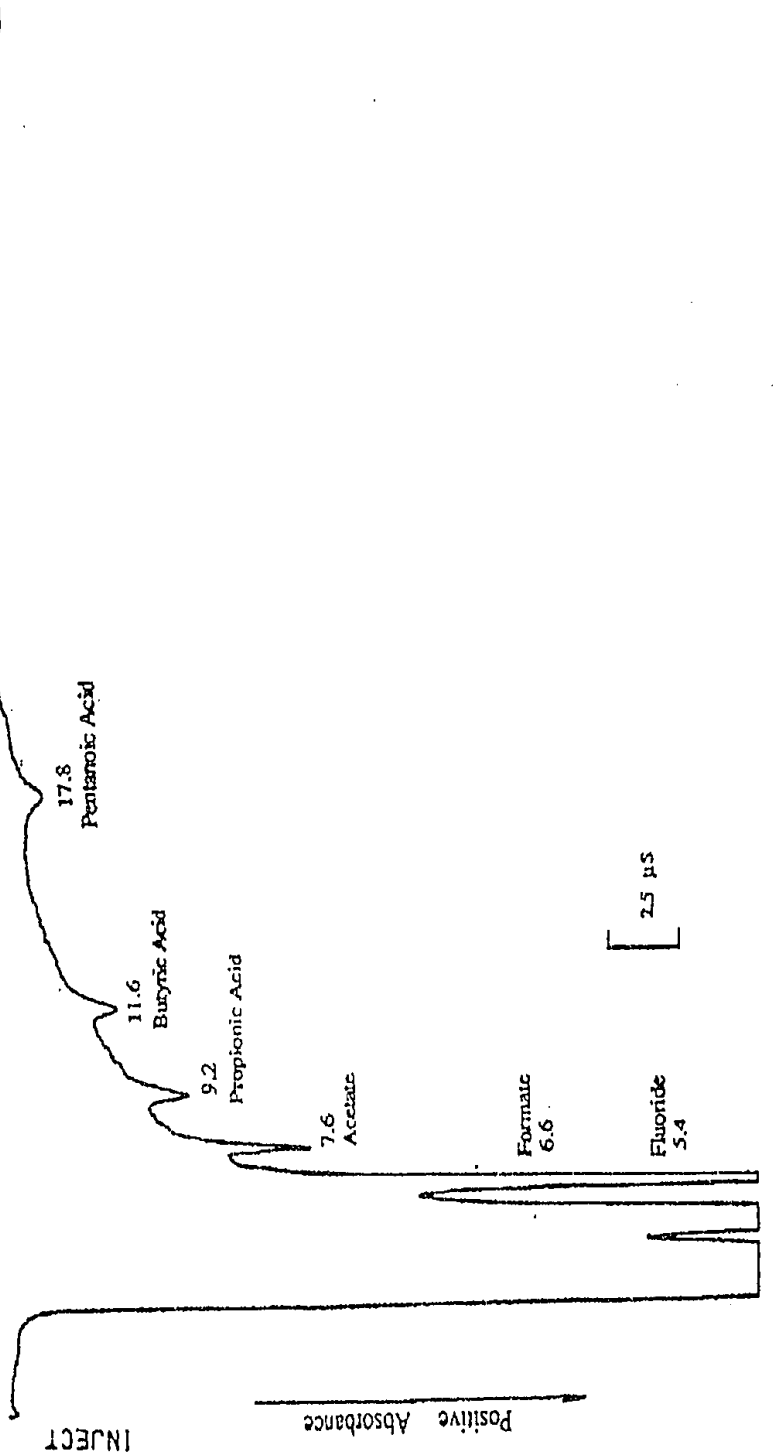

Figure 24. Chromatogram obtained with 0.75 mM naphthalenesulfonic acid as eluent by direct conductivity detection. Detection wavelength 294 nm. Other conditions as in Figure 20.

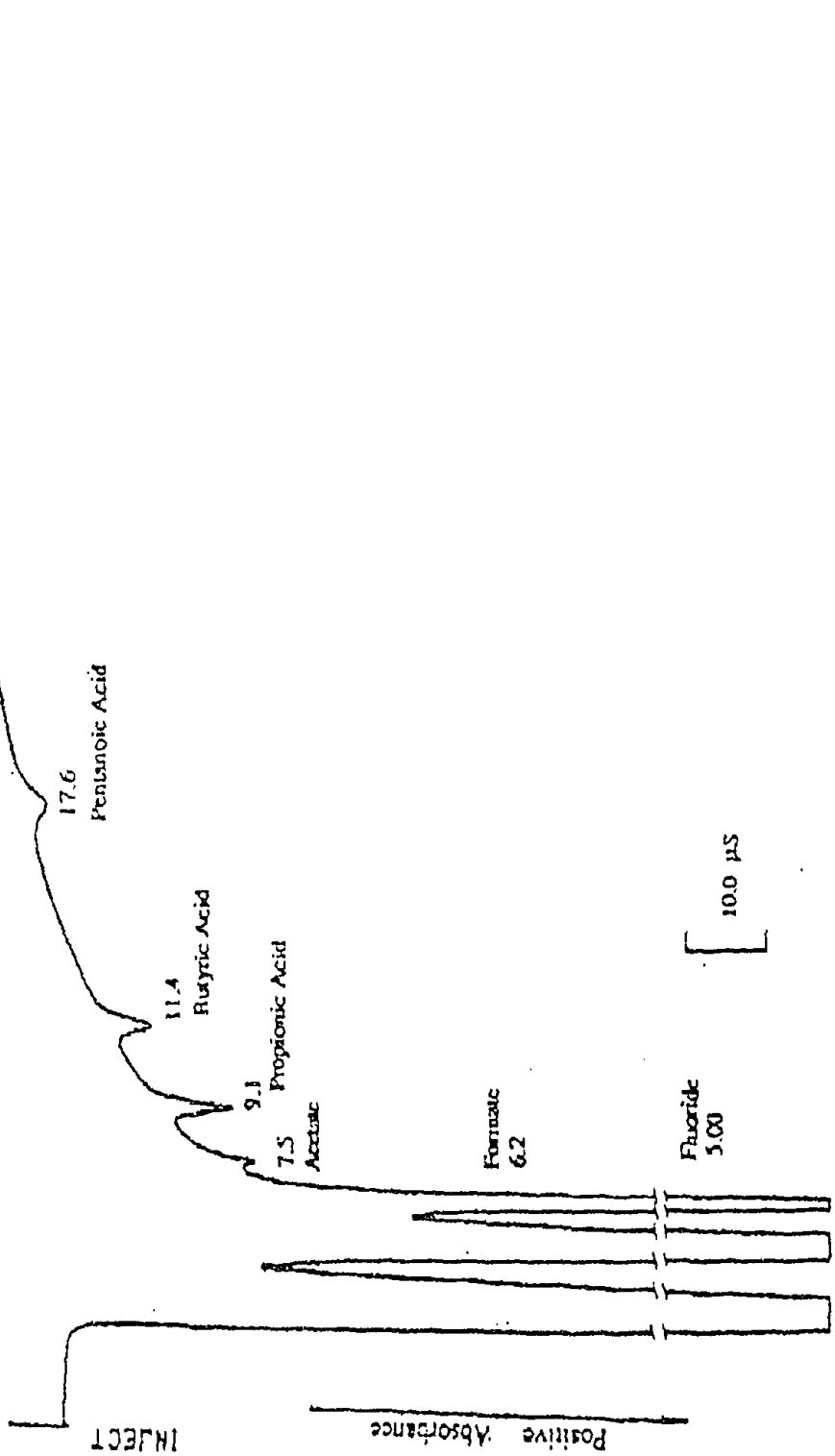

Figure 25. Chromatogram obtained with 0.75 mM toluenesulfonic acid as eluent by direct conductivity detection. Detection wavelength 235. Other conditions as in Figure 20.

Table 1. Distribution Coefficient for Carboxylic Acids Calculated from the Retention Data Shown in Figure 1.0-6.0.

		D_A					
Weak Acids	pKa1	MSA	ESA	OSA	CSA	NSA	TSA
Formic	3.75	0.00	0.03	0.00	0.07	0.00	0.00
Acetic	4.75	0.13	0.20	0 17	0.27	0.20	0.17
Propionic	4.87	0.73	0.73	0.70	0.70	0.73	0.70
Butyric	4.81	1.50	1.53	1.50	1.50	1.53	1.50
Pentanoic	4.82	3.50	3.60	3.53	3.40	3.60	3.50
Caproic	4.88	7.40	7.70	7 70	7.20	nd	nd
Heptanoic	4.89	15.8	nd	nd	nd	nd	nd
Caprylic	4.89	nd	nd	nd	nd	nd	nd

MSA - Methanesulfonic Acid ESA - ethanesulfonic Acid OSA - Octanesulfonic Acid nd - not detected
NSA - Naphthalenesulfonic Acid TSA - Toluenesulfonic Acid CSA - Camphorsulfonic Acid

Table 2. Detection Limits Obtained for the Carboxylic Acids and Inorganic Anions with Various Sulfonic Acid Eluents.

All values are expressed in parts per million (ppm) and are based on a direct injection of 20 µL aliquouts of a mixture containing 50 ppm of each of the solutes listed. The detection limits were calculated for a signal to noise ratio of 2.

Eluent	Detection mode	C1	C2	C3	C4	C5	C6	C7	C8	F^-	PO_4^{3-}
Methanesulphonic acid	UV	1.52	3.33	5.26	7.14	12.50	40.00	nd	nd	nd	nd
Ethanesulphonic acid	UV	1.75	3.45	5.00	7.14	11.11	50.00	nd	nd	nd	nd
Octanesulphonic acid[a]	UV	2.00	5.00	7.69	4.76	11.11	50.00	100.00	nd	nd	nd
	Cond.	1.33	4.54	14.28	28.57	33.33	100.00	nd	nd	nd	nd
Camphorsulphonic acid	UV	1.82	5.00	6.25	7.69	20.00	50.00	nd	nd	nd	7.14
Naphthalenesulphonic acid	Cond.	0.88	5.55	14.28	20.00	33.32	nd	nd	nd	1.35	nd
Toluenesulphonic acid	Cond.	0.71	25.00	10.00	16.67	50.00	nd	nd	nd	0.51	nd

a = free acid

nd = not detected

CHELERYTHRINE INHIBITS THE SECRETORY RESPONSE OF HUMAN BLOOD PLATELETS WITHOUT SPECIFICALLY INHIBITING PROTEIN KINASE C

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ABSTRACT

Chelerythrine (chloride) has previously been documented to be a potent and selective inhibitor of the serine threonine-specific protein kinase C (PKC). In this study, it was shown that 10 μM chelerythrine completely inhibited serotonin secretion and partially inhibited phosphatidic acid formation, in human blood platelets activated by thrombin, (1 U/ml). However, there was no effect on PKC activity, as assessed by the level of phosphorylation of the 47 Kd protein. Therefore, chelerythrine has been shown not to be a specific inhibitor of PKC and without specifically affecting PKC activity, is nevertheless capable of completely inhibiting platelet secretion, indicating that it may affect the signal transduction pathway responsible for pletelet secretion at a point downstream or independent of PKC.

INTRODUCTION

Platelet responses and stimuli are diverse, but it is emerging that their physiological activation is regulated by a distinct number of mechanisms, namely a receptor-mediated signal that is transduced through the membrane by guanine nucleotide-binding (G) proteins. The latter regulate specific effector systems, thereby modulating levels of intracellular second messengers whose activities lead to physiological responses. For example, putative G proteins regulate phospholipid hydrolysis by phospholipase-C (PLC), generating inositol-1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG). These second messengers respectively act as a mobilizer of Ca^{2+} from internal stores, and an activator of Ca^{2+}/phosphatidylserine-dependent serine/threonine protein Kinase-C (PKC). The former activates Ca^{2+}/calmodulin-dependent myosin light chain (MLC) kinase (Hathaway and Adelstein, 1979). Phosphorylated MLC (a 20 Kd protein) has actin-activated ATPase activity and assembles into filaments following interaction with the cytoskeleton (Fox and Philipps, 1982). This is thought to have a major role in the folding of the surface membrane and the contractile wave, responsible for centralizing secretory granules during spheration (White, 1974). These early events correspond to an initial low level of PLC and PKC

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activity. However, PKC activation occurs more slowly due to the prior requirement of priming with mobilized Ca^{2+} (necessary for shifting cytosolic PKC to a strategic position in the plasma membrane) and is probably not involved in the induction of shape change, but may participate in later stages or its maintenance (White et al., 1974; Siess et al., 1984).

For many stimuli, a second stage of activation occurs, which includes the formation of arachidonate from membrane phospholipids, by phospholipase-A_2 (PLA2) (Purdon et al., 1987). Released arachidonate is rapidly converted to biologically active metabolites (eicosanoids) by cyclooxygenase, generating prostaglandin endoperoxidases (EPs) such as G_2 and H_2 (Hamberg et al., 1974, 1975; Nugteren and Hazelhof, 1973; Detwiler and Huang, 1980) which aid inflammatory responses and are further metabolized to thromboxane A_2 (TxA_2) (Halushka et al., 1985). The formation of EPs and TxA_2 occurs just prior to secretion (Siess et al., 1984; Smith et al., 1973) in which they also play an important role (Siess et al., 1985). Their release, and the secretion of ADP from dense granules provide two efficient positive feedback loops (Rittenhouse-Simmons and Deykin, 1981; Malmsten, 1975) since they act as local hormones by binding to specific receptors, thereby triggering similar molecular events as other stimuli. They may be responsible for the shift from primary (reversible) to secondary (irreversible and secretion-dependent) responses (Siess et al., 1983) and for the increased activity of PLC and PKC observed during this transition (Siess et al., 1983). Also contributing to amplification is the synergistic behaviour frequently demonstrated by secreted agonists.

The understanding of mechanisms underlying platelet activation is increasing considerably but is by no means complete. It has been documented that PKC activation alone (via phorbol esters) is insufficient for secretion (Lapetina, 1985; Kaibuchi et al., 1983) but synergizes with Ca^{2+} (Siess and Lapetina, 1988; Nishizuka, 1984) and may exert negative feedback at PLC (Watson and Lapetina, 1985; Zavoico et al., 1985; Rittenhouse and Sasson, 1986). The present study attempted to investigate and further elucidate the role of PKC in the secretion response of fresh human blood platelets. The secretion of serotonin (5-hydroxytryptamine) was measured by monitoring the release of ^{3}H-serotonin from prelabelled platelets, and the involvement of PKC was reflected by the level of phosphorylation of the cytosolic 47 Kd protein. This widely accepted specific substrate of PKC (Imaoka et al., 1983) has been named PLECKSTRIN (platelet and leukocyte C-kinase substrate with the most probable phosphorylation site KFARKSTSIR), but its function and identity are unknown (Molina-Vedia and Lapetina, 1986). It has been suggested to be IP^3 phosphatase, responsible for negative feedback by PKC via IP_3 metabolism (Tyers et al., 1988; Conolly et al., 1986), though enzymatic activity can be partially separated from the 47 Kd protein. It has also been proposed that it functions as a lipocortin, which when phosphorylated by PKC, discontinues PLA_2-inhibition (Toqui et al., 1986), however, lipocortins only seem to be tyrosine-phosphorylated (Brugge, 1986).

It is evident that molecular events following receptor activation occur in a highly ordered manner, involving multiple interactions. A prerequisite to their elucidation and understanding are the discovery of drugs which are effective and specific inhibitors of specific components in signal transduction pathways. One such drug is chelerythrine (chloride), a 2,3,7,8-oxygenated benzo-[0]-phenanthridine alkaloid of MW 1188, isolated from *Zanthoxylum simulans* (Ko et al., 1990) and widely used in Asia as an antiplatelet agent. It has been previously documented to be a potent and specific inhibitor of PKC (Herbert et al., 1990) and would therefore be particularly useful in determining the complex and possibly bidirectional roles of PKC and hence contributing to the insight into platelet activation at the molecular level, also important for understanding general cellular signal transduction as well as thrombosis haemostasis.

MATERIALS AND METHODS

Platelet Preparation

Blood was drawn on the day of the experiment, by venipuncture from healthy aspirin-free volunteers. Approximately 50 ml blood were collected in a syringe containing 2 ml of 3.8% (w/v) sterile sodium citrate, as anti-coagulant, and approximately 7 ml of acid-citrate-dextrose (ACD) solution at 37°C (49 mM citric acid, 85 mM sodium citrate,110 mM glucose) were drawn up afterwards. The platelet-rich plasma (PRP) was obtained by centrifugation at 200g for 20 minutes at 25°C, and platelets were isolated from PRP by centrifugation at 1000g at 10 minutes, in the presence of 1 mg/ml prostacylin, which prevented spontaneous platelet activation by increasing the intracellular cAMP level. The platelet pellet was resuspended in 1 ml Tyrode's buffer at 37°C (138 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.36 mM Na_2HPO_4, 20 mM HEPES, 5 mM glucose, 1 mM $MgCl_2$, pH 7.3) containing 1 mM EGTA (to prevent aggregation by chelating external Ca^{2+}) and 10 uM indomethacin (to prevent positive feedback pathways by inhibiting cyclooxygenase) unless otherwise stated. Platelet number was determined by Coulter Counter and adjusted to the required density in Tyrode's buffer and challenged with various agents as described.

Serotonin (5HT) secretion with intact platelets

PRP was incubated with ^{3}H-5HT (0.5 uCi/ml) for 1 hour at 37°C, followed by the isolation of platelets as above. Aliquots of platelet suspension were prewarmed for 5 minutes in a 37°C non-shaking waterbath, under non-stirring conditions. After drug additions, the reaction period was initiated by adding thrombin and terminated by adding an equal volume of 6% glutaraldehyde/phosphate buffer solution. The mixture was microfuged at full speed for 60 seconds and an aliquot of supernatant was withdrawn for scintilation counting. Blanks (without drug additions) and total tissue samples (without drug additions and without centrifugation) were also prepared.

The % secretion of ^{3}H-5HT was calculated from the disintegrations per minute (dpm), using the following formula:

$$\% \text{ secretion} = \frac{\text{dpm (test)} - \text{dpm (blank)}}{\text{dpm (total tissue)} - \text{dpm (blank)}} \times 100$$

Phosphorylation studies with intact platelets

Platelets were prepared as described and resuspended in 1 ml Tyrode's buffer. They were incubated with 1-2 mCi ^{32}P for 1 hour at 37°C, washed and centrifuged at 1000g at 25°C for 10 minutes in the presence of prostacyclin, and resuspended to the required density. Aliquots were prewarmed in a 37°C non-shaking waterbath for 5 minutes under non-stirring conditions, before challenging with drugs.

Protein phosphorylation

After the reaction period, aliquots were withdrawn, added to an equal volume of reducing sample solvent and immediately boiled for 3 minutes, after the method of Laemlli (1970). Proteins

were separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 11% polyacrilamide mini-gels and protein bands were made visible by staining with PAGE-blue. Gels were dried under vacuum and exposed to autoradiography film either at room temperature or -70°C. After development, autoradiograms were used to identify protein bands of interest, which were cut out for scintillation counting to quantify ^{32}P incorporation.

Phosphatidic acid formation

After the reaction period, 250 ul aliquots were added to 940 ul of a 1:2 mixture of chloroform/methanol, in polypropylene tubes. 310 ul deionized water and 310 ul chloroform were added, and the mixture was centrifuged at 200 g at 25°C for 5 minutes. 200 ul of lower organic layer were extracted and concentrated by evaporating off the solvent overnight in a fume-cupboard. 50 ul chloroform were used to redissolve the lipids and the mixture was placed on a thin layer chromatography (TLC)-plate (Whatman LK60), by repeatedly applying 5 ul dots in the same position, allowing each application to dry. Lipids were separated by ascending chromatography, in a solvent which consisted of the upper phase of a 9:5:2:10 mixture of ethylacetate/isoctane/acetate/water, plus 1 ml acetate per 100 ml upper phase, after the method of Lapetine and Cuatrecasas (1979). An autoradiogram was prepared and bands corresponding to radioactively labelled phosphotidic acid were scraped and measured by scintillation counting to quantify ^{32}P incorporation.

RESULTS

Serotonin secretion and phosphatidic acid formation

Both serotonin and phosphatidic acid (PA) formation, induced by 1 U/ml thrombin, were inhibited by chelerythrine in a dose-dependent manner (Figures 1 and 2). With 10 uM inhibitor, secretion was fully blocked and extents of inhibition of PA formation were similar in the presence or absence of EGTA/indomethacin (43% and 33% inhibition respectively) (Figure 3).

300 nM B-phorbol dibutyrate (PdBu) alone, induced very little PA formation, as also observed with 10 uM chelerythrine alone (Figure 2). The latter also induced negligible serotonin secretion (Figure 1).

Phosphorylation of 47 Kd and 20 Kd proteins

100 nM - 10 uM chelerythrine caused insignificant changes in the phosphorylation levels of either protein, when platelets were stimulated with 300 nM PdBu (Figure 3, samples 2-5). However, when platelets were activated with 1 U/ml thrombin, 10 uM chelerythrine caused slight reductions (10% and 5% respectively) in phosphorylations of 47 Kd and 20 Kd proteins (Figure 4; compare samples 7 and 10). The amount of PdBu added was sufficient to mimic the degree of 47Kd phosphorylation that was achieved by thrombin, but that of the 20 Kd band was consistently above basal levels, albeit lower than for thrombin (in the range 37-119% above basal). In support of others (Chiang et al., 1981; Naka et al., 1983), this suggests that PKC activation alone is not only capable of 47 Kd protein phosphorylation, but also of MLC phosphorylation, hence revealing a limitation if this assay were used as a test of PKC inhibitor specificity.

DISCUSSION

The present study showed that concentrations of chelerythrine which blocked secretion and PA formation, had minimal effect on the phosphorylation of 47 Kd and 20 Kd proteins in thrombin-stimulated human platelets. Therefore, chelerythrine is not a selective PKC inhibitor and is of little or no use in studying the role of PKC in platelet activation.

Total inhibition of serotonin secretion and partial inhibition of PA formation by 10 uM chelerythrine were observed. PA formation is known to be dependent upon events leading to and including secretion (Nozawa et al., 1991). Furthermore, it has been previously shown to precede arachidonic acid production and parallel serotonin secretion, and has been used as an indicator of PLC activity (Watson et al., 1988). Taken together, the results suggest that chelerythrine exerts its inhibitory action at the level of PLC. Consistent with this and previous observations that 8.4 uM chelerythrine caused substantial inhibition of ADP release and thromboxane B_2 formation and inositol phospholipids breakdown in thrombin-stimulated rabbit platelets.

PLC inhibition would account for both total reduction of secretion in the absence of indomethacin (i.e. with participation of cyclooxygenase) and reduced PA formation, independent of cyclooxygenase activity (Figures 1 and 2). However, chelerythrine did not significantly affect PKC activity (Figure 3). One explanation is that the PLC inhibition required to prevent secretion could have been minimal, thus affecting DAG levels such that maximal PKC activation still occured. However, the reduction in DAG by inhibition of merely the PLC-B isoform could have been sufficiently counteracted by other processes yielding DAG, nominally phosphatidylcholine breakdown by the PLC-g isoform (which incidentally also lyses PIP_2) (Nishizuka, 1992), or vice-versa. The results simply imply that PKC activation is not the target for chelerythrine, and that platelet secretion can be inhibited at a point distinct from PKC. Unfortunately, this study does not shed any light on whether PKC has a regulatory role over PLC; others have shown this not to be the case.

It is interesting that MLC phosphorylation was not significantly affected; if PLC were indeed the target, a much greater extent of decline would be expected, implying here that if chelerythrine were to affect PLC directly, it would do so without affecting intracellular calcium supplies, suggesting that perhaps IP_3 levels are also unaffected. The results could thus also be explained by an inhibition or processes directly yielding PA including DAG-kinase (which phosphorylates DAG to PA), although implications of the inhibition of PA formation per se on platelet activation are unclear.

Alternatively, although secretion may require PKC activation, its regulation may have been completely independent thereof. The latter suggests that chelerythrine may block secretion by some mechanism which is distal to or independent of PKC activation, speculatively at the level of the secretion vesicle.

In conclusion, the role of PKC in secretion cannot be established from present studies because of the apparent non-selectivity and/or low potency of chelerythrine as a PKC inhibitor. Its apparent IC_{50} value in this study is around 1.5 uM. Herbert et al. (1990) obtained an IC_{50} value of 0.66 uM for inhibition of isolated rat-brain PKC by chelerythrine in a mixed micelle assay and showed that it bound the catalytic site of PKC. The different methodologies immediately explain the conflicting results presented here. However, despite possible problems in targetting the drug to sites of activated PKC, the present study showed that chelerythrine inhibits secretion and although its site of action is uncertain, a process independent of PKC may have been affected. Thus the search for a potent and specific PKC inhibitor— central to signal

transduction studies— continues.

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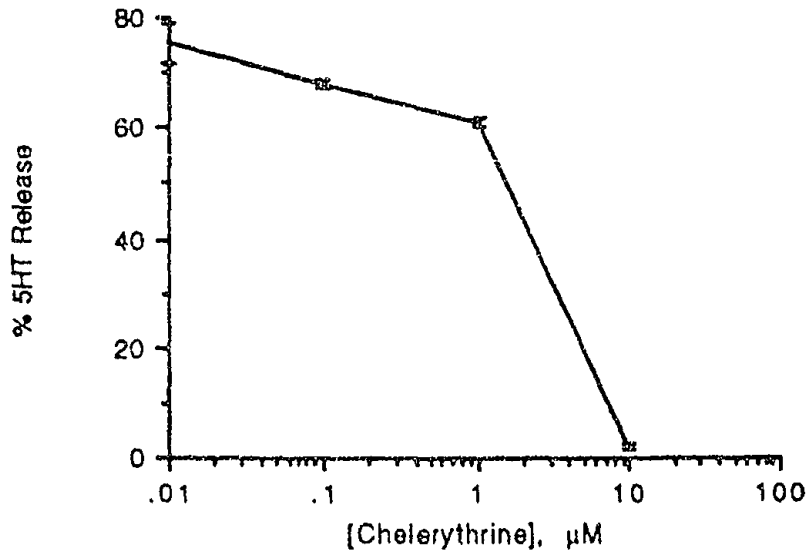

Figure 1. Platelets were labelled with ^{3}H-5HT and resuspended in Tyrode's buffer, in the absence of indomethacin, to a density of $2x10^8$/ml. Using a total volume of 500µl, 480µl aliquots of pre-warmed platelet suspension were incubated for 60 seconds with various concentrations of chelerythrine, and then challenged with 1U/ml thrombin for 60 seconds. This figure represents the dose-response curve for the inhibition of serotonin (5HT) secretion by chelerythrine. Each point represents the mean ± s.d (n=4). The ^{3}H-5HT secretion induced under these conditions, by thrombin alone was 69.4 ± 0.1% with 10 µM chelerythrine alone was 2.2 ± 1.5%.

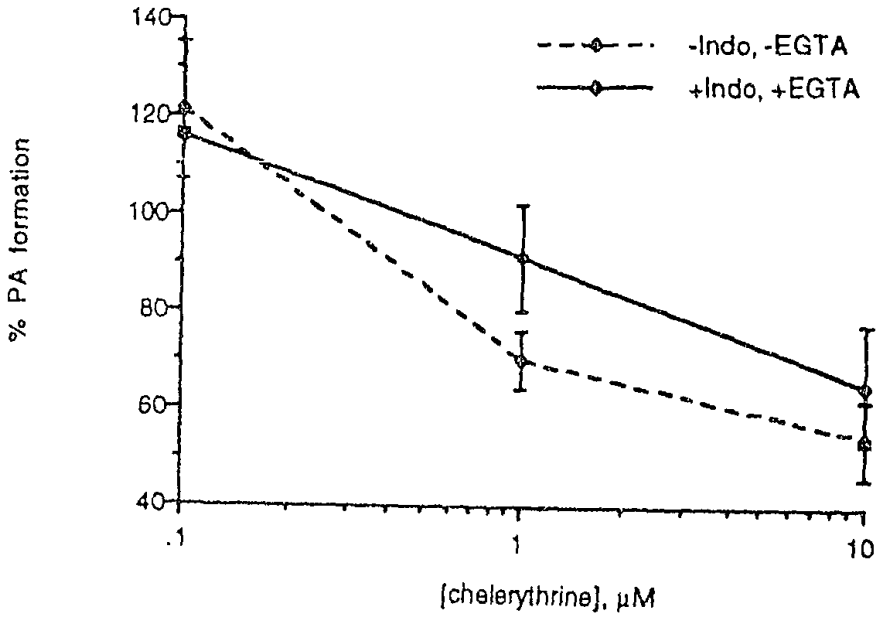

Figure 2. Platelets were (i) pre-labelled with 2 mCi/ml ^{32}P and resuspended in Tyrode's buffer in the presence of EGTA/indomethacin, or (ii) pre-labelled with 1mCi/ml ^{32}P and resuspended in Tyrode's buffer in the absence of EGTA/indomethacin, to a density of $2x10^8$ / ml. Using a total volume of 300µl, 288 µl aliquots of pre-warmed platelet suspension were incubated for 60 seconds with various concentrations of chelerythrine, and then challenged with 1U/ml thrombin for 60 seconds. 250µl aliquots were extracted for analysis of phosphatidic acid (PA) formation. The figure represents the dose-response curve for the inhibition of PA formation in the background cpm value. Each point represents the mean % ± s.d. (N=4). The presence and absence of EGTA/indomethacin (indo) and corresponded to 7361 and 2744 dpm respectively; basal PA levels were 262 and 643 dpm, respectively. In the absence of thrombin, 10 µM inhibitor alone gave 4 ± 1% PA production.

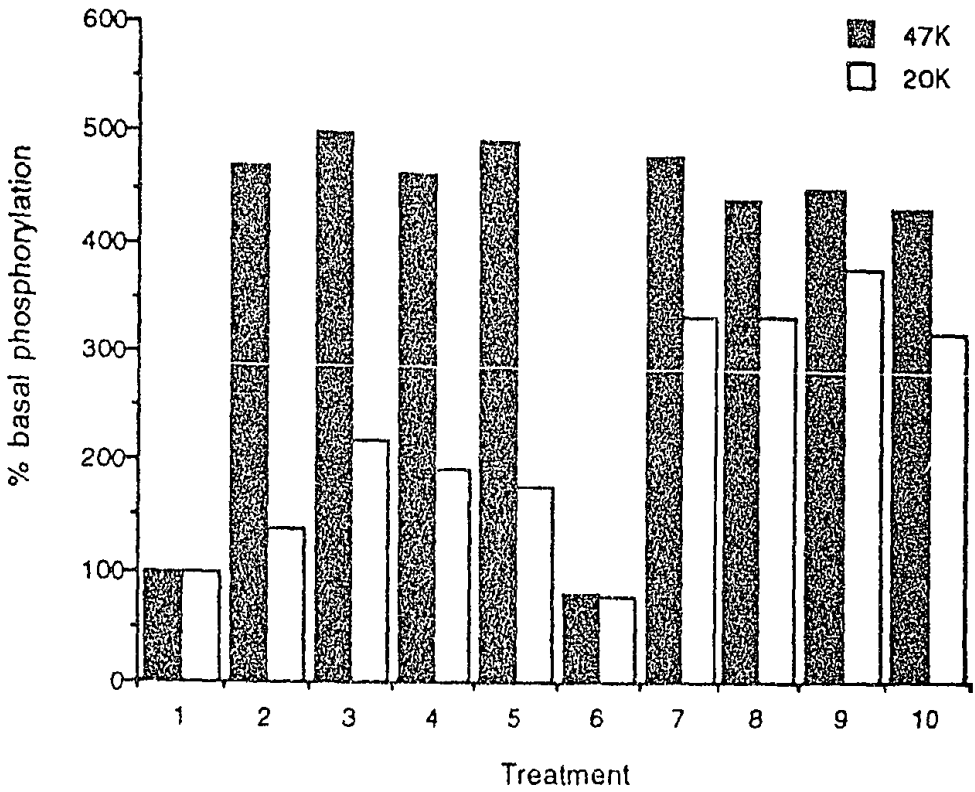

Figure 3. Platelets were prelabelled with 2 mCi/ml ^{32}P and resuspended in Tyrode's buffer, to a density of $2x10^8$ /ml. Using a total volume of 300µl, 288µl aliquots of pre-warmed platelet suspension were incubated for 60 seconds with various concentrations of chelerythrine, and then challenged with 1U/ml thrombin or 300 nM PdBu for 60 seconds. 50µl aliquots were extracted for analysis of protein phosphorylation. This figure represents a bar chart showing the effect of chelerythrine on the phosphorylation of 47K and 20K proteins. Results are expressed as % respective basal phosphorylation after subtraction of the background cpm value, for the autoradiogram presented. Basal phosphorylations were taken to be 100% and corresponded to 159 and 27 cpm for 47K and 20K, respectively.

Treatments: 1)basal, 2) PdBu alone, 3) PdBu + 100 nM inhibitor, 4) PdBu + 1 µM inhibitor, 5) PdBu + 10µM inhibitor, 6) 10 µM inhibitor alone, 7) thrombin alone, 8) thrombin + 100 nM inhibitor, 9) thrombin + 1 µM inhibitor, 10) thrombin + 1 µM inhibitor.

COTTON BOLLWORM IN THE PHILIPPINES: A REVIEW

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ABSTRACT

The cotton bollworm, Helicoverpa is one of the most serious pests in cotton . Helicoverpa can reduce seedcotton yield by 37 to 92% and cotton farmers spend 35 to 47% of their cash cost to control it. There have been deliberate attempts to develop control strategies towards effective management of this pest. Information available about Helicoverpa in cotton in the Philippines is reviewed in this paper. Its taxonomy, morphology, life history, life tables, ecology, phenology, economic importance, control measures and natural enemies are discussed.

INTRODUCTION

Insects compose the largest group in the animal kingdom. They consist about 80 percent of the total animal species numbering almost 20 to 50 million. About half of the total insect species are plant eaters (Southwood, 1977) but only some 100,000 plant eating species are potential pests as they attack the plant species of economic interest (Van Huis and Van Lenteren, 1992). Hargreaves (1948) listed 1326 species of insects in cotton and Gabriel (1977) noted that there are more than 60 species of arthropod pests that attack cotton in the Philippines. However, only about 14 species were reported to be capable of assuming alarming populations if appropriate control measure are not implemented (Cabangbang et. al., 1981). But recently, CRDI narrowed down the key pests to four: cotton leafhopper *Amrasca biguttula*, pink bollworm *Pectinophora gossypiella*, cotton flower weevil *Amorphoidea lata* and the cotton bollworm *Helicoverpa* complex (CRDI, 1988).

In this review, we attempt to give (a) the biology of *Helicoverpa*; and (b) its management and the potential role of natural enemies therein. Most of the literatures were from the Cotton Research and Development Institute, Batac, Ilocos Norte. Some were from masteral and doctoral theses of students from the University of the Philippines at Los Baños, College, Laguna, Philippines.

Taxonomy and Morphology

The cotton bollworm (corn earworm) was first described by Huebner in 1805 under the name *Noctua armigera*. Later, it was put under the genus *Heliothis* Ochsenheimer (1826) by Tritschke (Deang, 1971). Hampson (1903) transferred the species to the genus *Chloridea* Westwood and revived the specific name *obsoleta Fabr.* Its new name, *Chloridea obsoleta* had gained acceptance from *lepidopterists*. However, in 1939 Heinrich established *Heliothis armigera* as the proper scientific name for the insect (De Pedro, 1979). This move gained acceptance until

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Hardwick (1965) reported that this insect complex constitute a compact and well-defined group which differs significantly from *Dipsacea*. *Helicoverpa armigera* is currently being used to name the species, although some entomologists use the previous name *Heliothis armigera*. The genus *Helicoverpa* belongs to sub-family *Heliothidinae* and the family *Noctuidae* (*Phalaenidae*).

In the Philippines, the *Helicoverpa* complex has many vernacular names depending on the plant host it attacks. It is referred to as cotton bollworm in cotton, corn earworm in corn, tomato fruitworm in tomato, tobacco budworm in tobacco, sorghum headworm in sorghum (Gabriel, 1969) and pod borer in vegetable legumes.

The *Helicoverpa* complex constitutes a morphologically homogeneous group. Biological studies and notes on the insect in the Philippines have revealed the existence of several color forms (Otanes and Karganilla, 1940; Catan, 1958; Uichanco, 1959; Gabriel, 1969; Deang, 1971; Ramos and Morallo-Rejesus, 1976). However, several workers recorded the existence of *Helicoverpa assulta* (Guenee) (Capco, 1957; Hardwick, 1965; Ramos and Morallo-Rejesus, 1980 and *Heliothis* (*Helicoverpa*) *virescens Fabricius* (Silayan, 1938) aside from *Helicoverpa armigera*. Ramos and Morallo-Rejesus (1980) found that collected insects from tobacco had two distinct types of genitalia which correspond to *H. armigera* and H. Assulta. Deang (1971) described the morphology of the egg, larvae, pupa and adults of *Helicoverpa armigera*. He also made a detailed descriptions of the head, thoracic and pseudolegs, and chaetotoxy of the sixth-instar larva, and wing pattern and venation, head, legs and genitalia of the adults.

Helicoverpa as a Pest of Cotton

Helicoverpa, one of the most serious pests of cotton in the Philippines, attacks the fruiting structures like squares, flowers and bolls. The second larval instar of *Helicoverpa* damaged 1.7 to 2.0 squares daily while fourth and fifth instars, 3.7 to 4.7 squares (Campos and Orlido, 1978). Cummulative damage caused by two to three larvae on 10 plants from 40-120 days after plant emergence (DAE) significantly reduced seedcotton yield (Orlido, 1981). About 35-47% of the production costs goes to chemicals (almost entirely to *Helicoverpa* control) (Catudan and Rosario, 1993). Farmers spray their cotton crops eight to 15 times per growing season.

Life History and Life Tables

In the Philippines, the life cycle and developmental stages of *Helicoverpa* were investigated by Obien et al. (1985), Catan (1958), Deang (1971), Gabriel (1969) and De Pedro (1979) (Table 1). Its biology on several hosts and artificial diets was studied by Ramos and Morallo-Rejesus (1981) (Table 2).

Mortality factors of *Helicoverpa* on the various stages were natural enemies, diseases, physiological defects and insecticide treatments. *Trichogramma sp.*, *Sonolepsis germinata*, *Campoletis sp.* and *Carcella sp.* were recorded as natural enemies. Physiological defects were caused by feeding on nutritional-deficient host plant. Mortality of this pest during the early instars appeared to be very high often causing a population decline in future generations (Obien et. al., 1987 and Solsoloy et. al., 1994).

Ecology

Helicoverpa is extremely polyphagous and feeds on a wide variety of cultural crops. Deang (1971) listed 84 host plants in the Philippines (Table 3).

Female moth deposit eggs singly on squares, flowers, bolls, leaves and terminal buds at the upper-third portion of the cotton plant (Ugare et. al., 1986; Solsoloy et. al., 1994), but mostly on the first leaf (abaxial) below the terminal point of the main stem (Pascua, 1993). Earlier findings also revealed that *Helicoverpa* moth preferred to oviposit on the following excised plant parts in descending order: growing tips, flowers, bolls, squares and stems (Obien and James, 1990; Agustin, 1980).

The newly hatched larvae prefer to feed on the flowers especially the anthers and petals (Obien and James, 1990). However, the plant parts fed on also depend on where the eggs were deposited. Early instar larvae prefer to feed on succulent parts but they have the tendency to move down to the developed bolls as they grow older. Most of the larvae were confined to the upper third portion from 43 to 64 DAE, the middle-third portion from 78 to 85 DAE, and the upper-third portion from 92 to 106 DAE (Solsoloy et al., 1993).

The larvae prefer to penetrate into the generative organs of the host plant. This causes the loss of squares, flowers or young bolls. If reproductive parts are lacking, they feed on growing leaves (Schmutterer, 1978).

Helicoverpa preferred to oviposit more on tomato and pigeonpea (Mangasep and James, 1988), and corn and tobacco (Querubin et al., 1986) than on cotton. However, Obien and James (1988) noted that Abutilon indicum was equally preferred. *Helicoverpa* preferred tomato as host at an early stage of the cotton crop (35-63 DAE) while pigeonpea was preferred at the later stages (Mangasep, 1989).

Phenology

The peak occurrence of *Helicoverpa* varies according to growing areas. It is also influenced by the crop stage (Obien, 1987; Suharto, 1989) and crop diversity in the area, even more than environmental factors like rainfall, relative humidity, air temperature and sunshine duration (Solsoloy et al., 1994). However, Eliarda (1993) claimed that egg deposition is positively correlated with temperature, relative humidity, lunar position and extent of cloudiness. De Asis (1985) also found that the abundance of eggs and larvae is influenced by environmental factors.

Helicoverpa density in cotton increased as the crop progressed from seedling to bolling stage and decreased towards flowering stage (Repoyo and Embuido, 1992). The highest larval population coincides with the peak reproductive stage of the crop in different cotton growing areas of the Philippines (Bulaeso, 1978; De Asis, 1985; Solsoloy et al., 1994). However, peak occurrence of *Helicoverpa* in late planted cotton (November to December) occurred in an earlier stage of cotton growth than in early planted cotton (August to October) (Orlido, 1985; De Asis, 1985; Obien, 1987; Solsoloy et. al.,1994). This is because early planted cotton becomes a source of infestation of late planted cotton especially when they are planted adjacently. Female moths prefer to oviposit on late-planted cotton during the peak reproductive stage when early planted cotton is already near maturity.

Control Measures of *Helicoverpa* in Cotton

Cultural Control

Cultural control is one of the basic components of *Helicoverpa* control and the management of fertilization rates is one of them. With a higher nitrogen level 220 kg N/ha than the recommended rate of 75-100 kg N/ha, the *Helicoverpa* population significantly increased (Ugare, 1985; Cimafranca, 1993; and Damo, 1994). In order not to surpass the recommended level it is

necessary to avoid excessive and luxuriant plant growth which creates more oviposition by Helicoverpa. However, different plant densities did not affect *Helicoverpa* population per area (Ugare, 1985).

The use of trap crops like tomato and pigeonpea (Mangasep and James, 1988), and corn and tobacco (Querubin et. al., 1986) can help to lower the *Helicoverpa* population on cotton. Planting a row of trap crop in cotton fields at an interval of 15-20 rows of cotton can divert the *Helicoverpa* away from the cotton. Cotton intercropped with corn and sorghum had a significantly lower number of *Helicoverpa* eggs and larvae than cotton monoculture, resulting in a lower number of damaged squares, flowers and bolls (Suharto, 1989). Further, cannibalism occurs among *Helicoverpa* larvae within the trap crop row especially at high population levels (Gergon and Gergon, 1981). The trap crops may also serve as habitat for natural enemies.

Cotton fields must be planted within the shortest possible time in a production cluster. This practice limits the proliferation of the pest. Early planted cotton is the source of infestation for late planted cotton (Obien, 1987).

The variety CRDI-1 is susceptible to *Helicoverpa* but resistant to cotton leafhopper. The delay of insecticide application during the early stage of the crop helps to preserve the natural enemies (Pascua, 1989).

Crop Pest Monitoring System

An unbiased monitoring system for both crop and pest is a prerequisite to rational pest management. Pest surveillance based on sequential sampling from 21 to 120 DAP prior to insecticide application reduced chemical sprays (Solsoloy et al., 1995). For *Helicoverpa*, 20 sample plants are selected randomly in the cotton field and these are observed for the presence or absence of eggs or larvae in the terminal point or in the upper 20 cm of the plant's main stem including leaves, squares, flowers, and bolls. Infested plants are summed-up and compared to the critical pest level (CPL) for *Helicoverpa*, i.e. for terminal points with larvae or eggs at 28-56 days after planting (DAP), three terminal points with larvae or eggs at 57-112 DAP, and three secondary branches with larvae or eggs at 113 onwards.

Biological Control

Biological control in the Philippines dates back to 1849 when the Spanish governor Juan Martinez introduced an insectivorous bird to control migratory locusts (Baltazar, 1980). This bird also fed on lepidopterous insect pests.

Helicoverpa natural enemies were surveyed and identified, the efficacy of some natural enemies was evaluated, and their use was made compatible with control measures like chemical and cultural control (Adalla and Pascua, 1987; Cahatian, 1990). A list of natural enemies of *Helicoverpa* is given in Table 4.

Parasitization of *Helicoverpa* eggs by *Trichogramma chilonis*, *T. chilotraea* and *Trichogrammatoidea bactrea bactrea* was evaluated in the field (Famoso and Alba, 1990), and that by *T. chilonis*, *T. chilotraea*, *T. evanescens*, *Tr. bactrae bactrae* and *Tr. cojuangcoi* in the laboratory (Famoso and Medina, 1991). *T. chilonis* and *T. chilotraea* were the most dominant egg parasitoides of *Helicoverpa* (Cadapan, 1986). They preferred newly laid *Helicoverpa* eggs (Torreno, 1982; Gonzales, 1991).

An efficient and cheap mass rearing technique of *Trichogramma* has been developed using *Corcyra cephalonica* (Cadapan, 1986; Famoso and Gonzales, 1986; Medina, 1980; Medina and

Cadapan, 1981) and *Sitotroga* (Gruber et al., 1992) as hosts. Cacayorin et al. (1993) and Gruber et al. (1992) discussed the procedures using the *Corcyra* and *Sitotroga* as hosts respectively.

Trichogramma can be released in the field using either pupa or emerging adults at 5 P.M. or 8:30 A.M., respectively (Cacayorin, 1992). When laboratory reared Trichogramma chilonis was released in the cotton fields at the rate of 67,000 - 80,000 adult parasitoids per release twice a week, egg parasitization rate was 50 to 96 percent (Famoso, 1988;1989). Release of *Thrichogramma* integrated with synthetic insecticide to control *Helicoverpa* had a seed cotton yield comparable to the treatment of using insecticide alone (Perpetua, 1987), and could reduce to the cost of chemical application by 21 percent (Solsoloy et al., 1995a) or 26 percent (Teruel and Jarbadan, 1990). However, if cotton fields have a high infestation of *Helicoverpa*, the use of *Trichogramma* cannot get the population below the critical pest level (Mangasep et. al., 1992). Furthermore, Thrichogramma is sensitive to pyrethroids and cannot be released in the field just before and after pyrethroid application (Cacayorin and Solsoloy, 1993).

To control *Helicoverpa* the use of *Bacillus thuringiensis* alone or in combination with other measures has been studied. Padua et. al. (1982) isolated the bacterium from soils collected from various regions of the Philippines. Damo (1988) also collected Bacillus sp. from soils from different cotton growing areas in the Philippines.

Adult *Helicoverpa* fed with 10 percent sucrose solution + *B. thuringiensis* isolates had a shorter life span, and fecundity was reduced (Damo, 1991). In using pathogenicity test, Nomorea rileyi caused 40 percent infection in the laboratory (Damo, 1993).

Commercial formulation of B. *thuringiensis* had been successfully used against *Helicoverpa* (Pojas, 1984; Layaoen, 1979; Tandan and Nillama, 1987; Damo, 1990). However, it is only effective when the *Helicoverpa* population is at a low level or occurs at an early stage of the cotton crop (Damo, 1992). A combination of this microbial insecticide with half the recommended rate of synthetic insecticides can control *Helicoverpa*. However the efficacy was lower than the recommended synthetic insecticides (Damo and Solsoloy, 1993).

Oxyopes javanus preferred the second and third instar larvae and consumed an average of 2.5 or 1.5 larvae per day when starved or unstarted, respectively (Cacayorin et. al., 1993).

Resistant Cotton Varieties

Gossypium barbadense cultivars and the *G. hirsutum* cultivar HGBR 8N were identified as resistant to *Helicoverpa* (Pascua, 1992). However, at present, no resistant variety is recommended for commercial cultivation.

Chemical Control

Chemical control is the last remedy in *Helicoverpa* control. If other control measures do not work, farmers always resort to the use of insecticides.

Researchers screened chemical insecticide application rate and duration of the intervention (Adalla and Pascua, 1987). The Cotton Research and Development Institute (1993) recommends certain insecticides at specific growth stages of the cotton plant for *Helicoverpa* control to prevent the pest from developing insecticide resistance (Cacayorin and Solsoloy, 1992). Insecticides especially Endosulfan and Deltamethrin, which are commonly used by farmers, should only be used for one or two generations.

Likewise, botanical pesticides were tested to substitute synthetic pesticides. Extracts from sweet flag (*Acorus calamus*) were effective as antifeedant for *Helicoverpa* larvae (Solsoloy, 1985).

Jatropha curcas oil extract had an insect regulator effect on *Helicoverpa* as a juvenile hormone mimic. *Helicoverpa* larvae topically applied or fed with oil treated diet developed into larval-pupal intermediates or abnormal adults. Normally developed adults have reduced fecundity or morphological defects in the ovarioles such as disintegrated oocytes and a reduced number and size of the spermatozoa (Solsoloy and Rejesus, 1992). However, field trials of the *Jatropha* oil extract had apparently inferior performance to the IPM technology for *Helicoverpa* even if integrated with *Trichogramma* (Solsoloy et. al., 1995).

Natural Enemies of Helicoverpa

Natural enemies like parasitoids, predators and pathogens play an important role in regulating insect pests populations. The presence of biological agents of *Helicoverpa* in the Philippines have been reported by some authors. However, literature on the life histories and effectiveness of the Philippine species is scanty and research efforts for the biological control of this pest are inadequate and fragmented.

Only 16 publications refer to natural enemies and most of them deal with *Trichogramma spp.* Another 30 unpublished works refer to laboratory studies, mass rearing techniques, identification, efficacy of the natural enemies in the field, and integration of biological control with other control measures. Baltazar (1962; 1964; 1966) published articles on the genera of parasitic Hymenoptera in the Philippines.

Parasitoids

The earliest records of *Helicoverpa* parasitoids in the Philippines concerns *Microplitis manilae* (Ashmead, 1904 and Edrozo, 1918). They and Divina and Irabagon (1976), Santhoy (1980) and Cacayorin et. al., (1993) reported the braconid, *Microplitis manilae* (Ashmead) as a parasitoid of *Helicoverpa* larvae. Other recorded parasitoids of *Helicoverpa* are presented in Table 4.

Baltazar (1963) listed 48 beneficial organisms introduced in the Philippines from 1850 to 1960 including four parasitoids of *Helicoverpa, viz: Cardiochiles nigriceps, Campoletis perdistinctus, Trichogramma japonicum and T. australicum (chilonis). Cardiochiles nigriceps* and *Campoletis perdistinctus* were sent by Prof. T. Gardner of U.S. Department of Agriculture upon the request of Dr. H. Townes and received by the Bureau of Plant Industry in August 1953 (Gardner, 1957 cited by Baltazar, 1963). However, rearing of the parasitoids in the laboratory in Manila was not successful and no adults were released in the field. *T. australicum (chilonis)* was introduced in the Philippines on July 2, 1954 from Formosa by Dr. G. Merino (Delfindo, 1959; 1935 cited by Baltazar, 1963). This *Trichogramma sp.* and *T. japonica* were established as *Helicoverpa* parasitoids in the country.

Cahatian (1990) discussed in her paper the diagnostic characters, description, stage of host parasitized and the parts of the Philippines where these parasitoids are located. Likewise, De Cock (1993) studied the bionomics of *Trichogrammatoidea cojuangcoi.*

Predators and Pathogens

Few arthoropods are reported as predator and two pathogens are attacking *Helicoverpa* (Table 4).

Evaluation of Natural Enemies of Helicoverpa

Few studies were conducted in the Philippines to evaluate the effectiveness of the natural enemies to control *Helicorverpa*. This concerned *Trichogramma spp.* as control measure and degrees of parasitization of naturally occurring beneficial insects in the field.

a. Parasitoids

Usually egg, larval and pupal stages of *Helicoverpa* are collected and brought to the laboratory for parasitoid emergence (Cahatian, 1990; Cacayorin et. al., 1993; Pascua and Pascua, 1995; Famoso and Alba, 1990). Estimating the abundance of adult parasitoids, which is a more difficult task, has hardly been carried out.

Helicoverpa eggs

Helicoverpa eggs were collected and placed individually in glass vials then brought to the laboratory for further observation. Parasitization was indicated by blackening of the eggs. The ratio of number of parasitized eggs and number of eggs collected is taken as the percentage of eggs parasitized (Pascua and Pascua, 1995). This procedure underestimates the degree of parasitization. The collected eggs have a lower chance of being parasitized due to a shorter exposure period compared to eggs parasitized in the field. Bringing the eggs to the laboratory also depletes the parasitoid population in the field.

A better procedure, although more laborious, is proposed. A number of plants in the field are tagged and examined for the presence of *Helicoverpa* eggs. Black eggs are recorded separately from the white eggs. White eggs are marked with cotton threads then examined after three days whether they have blackened or hatched into larvae. Black eggs from the first observation and after three days are summed up. The degree of parasitization is determined by the ratio of black eggs and the total number of eggs present in one sampling period.

Helicoverpa larvae and *pupae*

Larvae and pupae, collected in the field and brought to the laboratory, were placed individually in plastic cups covered with chrome wire screen and fed with the natural host or artificial diet. Each larva was observed daily for parasitoid emergence The emerged parasitoids were preserved in alcohol for identification. The degree of parasitization was determined using the ratio of number of parasitized larvae/pupae and total number of larvae/pupae collected (Cahatian, 1991; Cacayorin et. al., 1993).

However, van den Berg (1993) recommended to dissect field-sampled hosts to avoid death of the larvae during the rearing period. However, this procedure is time consuming and early instar parasitoids maybe overlooked. He also identified four major sources of errors and recommended some techniques to avoid or limit these errors:

1. Host-age specificity of parasitoids. Host stage of attack and emergence should be known for each parasitoid species; the percentage parasitization should be calculated separately.
2. Exposure period of susceptible host stage. The host should be sampled after the stage susceptible to attack but prior to the stage of parasitoid emergence.
3. Change of host stage development. Some larval and pupal parasitoid retard the development of their host. A partial solution is to place cohorts of a particular life stage into the field for subsequent monitoring.
4. Mortality of parasitized hosts. The probability of sampling error is especially high when parasitized hosts are more sluggish than healthy hosts.

Likewise, Seymour and Jones (1990) recommended a corrected parasitization rate if the relative development times of the parasitized and parasitized are known, that is:

$$\text{corrected parasitization rate} = 1 - (1 + k)\, S/2$$

where k is the ratio (development time of parasitized instar)/ development time of unparasitized instar); and S is the proportion of unparasitized larvae in the sample.

Further, the proper denominator for measuring parasitization should be the stages of hosts which are subject to parasitization (van Driesche, 1983).

Experiments with cages were used to evaluate the frequency of releases of two *Trichogramma* species by checking percent parasitization of *Helicoverpa* eggs (Famoso and Alba, 1990). Percent parasitization, larval counts, seedcotton yield and net profit were used to evaluate the effect of *Trichogramma* treatment alone and in combination with chemicals (Perpetua, 1987; Famoso, 1988; Mangasep et. al., 1992; Solsoloy, et al., 1995).

b. Predators and Phatogens

The evaluation of the effectiveness of predators and pathogens to control *Helicoverpa* has been dealt with in Control Measures of *Helicoverpa* in Cotton.

Recommended Integrated Pest Management (IPM) for *Helicoverpa*

Integrated pest management aims to lower pest population below the economic injury level. Uncontrolled pest populations may cause substantial damage to crops and high cost of pest control. Proper management therefore is necessary to ensure high yield, good quality harvest and high profit. The Cotton Research and Development Institute (1993) recommends the following components:

1. Plant cotton within the shortest possible time in a production cluster.
2. Fertilize cotton plants with recommended levels.
3. Plant trap crops like tomato, tobacco or corn in every 15-20 rows of cotton.
4. Use cotton variety CRDI-1
5. Monitor the pests weekly.
6. Release laboratory reared *Trichogramma chilonis* at the rate of 67,000 parasitoid/ha twice a week from 50 DAP.
7. Spray commercial formulation of Bacillus thuringiensis at the early growth stage of the crop (43-63 DAP).
8. Spray the recommended synthetic insecticide when the *Helicoverpa* population reaches the critical pest level.

This IPM package of technology for *Helicoverpa* has been very efficient in reducing the damage thereby producing high yield (Solsoloy et al., 1995). However, the only disadvantage is the high input for chemicals. This can be improved by significantly reducing the frequency of insecticide spraying by conserving and enhancing naturally-occurring beneficial insects. This technology not only reduces the production inputs but also preserves the biodiversity of the ecosystem.

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Table 1. Developmental time (days), longevity, fecundity and other biological data of *Helicoverpa* in the Philippines under laboratory and field conditions.

Stage	Field	Laboratory			
	Obien et al. (1985) Mean + SD	Catan (1958)	Deang (1971) Mean + SE	De Pedro (1979) Mean + SD	Gabriel (1969)
Egg	3.2 + 1.40	2 - 5	3.6 + 0.10	2.0 + 1.00	2 - 5
Larva					
1st Instar	3.8 + 1.40	2 - 5	2.8 + 0.10	3.8 + 1.25	-
2nd Instar	4.6 + 2.15	2 - 4	2.0 + 0.11	4.8 + 0.75	-
3rd Instar	3.3 + 1.12	2 - 4	2.2 + 2.21	4.0 + 1.00	-
4th Instar	3.2 + 1.15	2 - 4	2.3 + 0.13	3.5 + 0.50	-
5th Instar	5.1 + 1.14	2 - 5	2.3 + 0.14	4.0 + 1.00	-
6th Instar	4.0 + 1.41	4 - 11	5.8 + 0.30	5.0 + 1.00	-
Subtotal	24.4 + 8.37	17 - 25	17.3 + 2.99	25.0 + 5. 50	17 - 24
Pupa	9.8 + 0.81	12 - 14	10.8 + 0.16	11.0 + 3.50	12 - 14
Adult	5.1 + 0.40	-	10.3	10.0 + 7.00	
Total	42.5 + 10.99	29 - 41[1] 34 - 45[2]	42.0 + 3.15	47.5 + 17.00	34 - 45
No. of eggs laid/female		249 - 2746			200 - 2000
No. of generation/year		8.0			

[1]Male
[2]Female

Table 2. Biology of *Helicoverpa armigera* (Hubner) on several hosts in the Philippines.

Parameters	Semi-synthetic diet				Natural Host			
Yellow	Green Mongo	Corn Mongo	Cotton	Cotton	Tomato Control	Tomato	Tobacco Control	
	(1)*	(1)*	(1)*	(2)*	(1)*	(3)*	(1)*	(1)*
% of insects with complete life cycle	71.7	80.0	88.3	30.0	21.7	26.7	48.3	16.7
Duration of egg Stage (days)	2	2	2	2	2	2	2	2
Duration of larval period (days)	32.3	31.6	28.3	31.8	29.4	40.4	38.2	33.4
Weight of 6th Instar larva (mg)	209.8	215.1	214.8	225.8	148.8	93.2	112.8	96.7
Number of larval Instars	6	6	6	6	6	8	7	7
Pupal weight (mg)	349.2	347.7	412.3	309.2	290.5	272.4	261.6	275.2
Pupal period (days)	11.7	11.6	11.8	11.7	15.2	11.8	20.8	13.4
Adult fecundity (mean) of eggs/female	491.1	465.1	680.5	138.2	523.2	551.5	538.3	396.0
No. of reproductive days	6.0	8.2	5.8	3.2	6.6	5.2	8.0	8.0
Adult weight (mg)	172.1	169.8	213.3	141.2	181.7	152.9	137.9	167.5
Adult longevity (days)	9.2	10.7	8.2	5.8	7.8	8.5	12.1	11.0

[1]Insects were collected from corn plants
[2]Insects were collected from cotton plants
[3]Insects were collected from tomato plants

Source: Ramos and Morallo-Rejesus (1981)

Table 3. Host plants of *Helicoverpa* in the Philipines (Deang, 1971).

Host	Common Name	Reference
Amaranthaceae		
Amaranthus hybridae L.	Princes-Feather	Parsons, 1939
A. thunbergii Moq.		Parsons, 1939, 1940
Capparidaceae		
Cleome monophylla		Parsons, 1939, 1940
Chenopodiaceae		
Chenopodium hircinum Schrad		Parsons, 1939
C. murale L.		Parsons, 1939, 1940
Compositae		Parsons, 1939
Bidens pilosa L.	Sunflower	Parsons, 1940
Helianthus annuus L.	Lettuce	(Catan) 1958
Lactuca sativa		Parsons 1939
Sonchus asper L.		Parsons 1940
S. oleraceae L.		Parsons 1939
Tagetes minuta L.		Parsons 1939; 1940
Xanthium pungents Willd.	Zinnia	Personal Communication
Zinnia elegans Jacq.		with Dr. C.H. Baltazar
Cruciferae	Cabbage	Esguerra et al., 1969
Brassica oleraceae L.	Collard	(Catan) 1958
B. oleraceae var. acephala		
Cucurbitaceae	Wax gourd	Capco 1957; Deang 1969
Benincasa hispida (Thunb)	Watermelon	Capco 1957; Deang 1969
Citrullus lunatus (Thunb)	Melon	Capco 1957; Deang 1969
Cucumis melo L.	Cucumber	Capco 1957; Deang 1969
Cucumis sativus L.		Parsons 1940
	Winter squash	Parsons 1940
Cucurbita maxima Duch		Capco 1957; Deang 1969
C. moschata Duch ex Poir		Parsons 1940
C. pepo L.	White-flowered gourd	Capco 1957; Deang 1969
Lagenaria siceraria (Mol.) Standl.	Sponge gourd	Capco 1957; Deang 1969
Luffa cylindrica L.	Bittermelon	Capco 1957; Deang 1969
Momordica charantia L.	Chayote	Capco 1957; Deang 1969
Sechium edule (Jacq.)		
Euphorbiaceae		Parsons 1939
Acalypha segetalis Mull-Arg	Castor oil	Capco 1957; Wene 1955
Ricinus cummunis L.		
Gramineae	Oats	Cotes 1889; Fletcher 1920
Avena spp.		
	Sorghum	Esguerra et al. 1959;
Sorghum bicolor		Parsons 1940; Dahma et al. 1955;
	Hegari	Bailey et al. 1968; Kinzer et al. 1968

Table 3 con't. . . .

Sorgum sp.	Barley	Hardwick 1965
Hordeum sp.	Wheat	Parsons 1940
Triticum vulgare Vill.	Corn	Baltazar 1968
Zea mays L.		Capco 1957; Viado et al. 1957; 1958; Esguerra et al. 1959; (Catan 1958); Parsons 1940; Hardwick 1965
Labiatae		
Hoslundia opposita Vahl. var. decumbers Bakh		Parsons 1939; 1940
Leucasmartinicensis Ait.		Parsons 1939
Ocimum americanum L.		Parsons 1939; 1940
Onthosiphom serratum Schlech		Parsons 1939
Leguminosae	Peanut	
Arachnis hypogeae L.	Pigeonpea	Capco 1957
Cajanus cajan Mili sp.		Capco 1957 Deang 1969
Cajanus Indicus Spring	Chickpea	Hardwick 1965
Cicer arietinum L.	Sunhemp	Fletcher 1929; Parsons 1940
Crotolaria juncea L.	Hyacinth bean	Parsons 1940
Dolichos lablab L.		Capco 1957; Deang 1969; Parsons 1940; Hardwick 1965;
	Soybean	Matthews 1966
Glycine max L.	Wild pea	Capco 1957
Lathyrus sativus L.	Alfalfa	Hardwick 1965
Medicago sativa L.	Pea	Parsons 1940; Sloan 1945
Pisum sativum L.		Capco 1957; Deang 1969; Parsons 1940;
	Tepary bean	Matthews 1966
Phaseolus acutifolius		Parsons 1940;
	Mungbean	Hardwick 1965
P. aureus Roxb.		Capco 1957; Deang 1969;
	Lima bean	Parsons 1940
P. lunatus L.		Capco 1957; Deang 1969;
	Snap bean	Parsons 1940
P. vulgaris L.		Capco 1957; Deang 1969
Sesbania sp.		New record observed at
	Winged bean	IRRI multiple cropping
Tetragonolobus purpureus Moench		Capco 1957; Deang 1969
	String bean	
Vigna sinensis (Stick) savi ex Hassk (sesquipedalis group)		Capco 1957; Deang 1969 Esguerra et al. 1969 (Catan 1958);
	Cowpea	(Fontanilla 1959)
V. sinensis (Stick)		Capco 1957; Deang 1969; Parsons 1940
Liliacea	Onion	
Allium cepa L.	Garlic	Capco 1957; Deang 1969

Table 3 con't. . . .

	A. sativum L.	Garden asparagus	Capco 1957; Deang 1969
	Asparagus officinalis L.		(Catan 1958)
Linaceae		Flax	
	Linum sp.		Parsons 1940
Malvaceae			
	Abutilon indicum		Parsons 1940
	A. soneratianum	Cotton	Parsons 1940
	Cassavalum spp.		Capco 1957; Esguerra et al. 1959;
		Okra	(Catan 1958); Thomas 1931;
	Hibiscus esculentus L.		Hardwick 1965; Matthews 1966
			Capco 1957; Bautista et al. 1957;
		Roselle	Deang 1969;
	H. sabdariffa L.		(Catan 1958)
	Malvstrum tricuspidatum		Capco 1957; Deang 1969
	A. gray		Parsons 1939; 1940
	Sida rhombifolia L. var riparida Burtt Davy		Parsons 1939
Papaveraceae		Opium poppy	
	Papaver somniferum L.		
Pinaceae		Monterey pine	Fletcher 1920; Hardwick 1965
	Pinus radiatus D. Don.		
Rosaceae		Rose flower bud	Hardwick 1965
	Rosa spp.	Lupine	
	Rubus sp.		(Catan 1958)
Rutaceae		Citrus	Nel 1961
	Citrus spp.		
Solanaceae			Parsons 1940; Jones 1934;
	Capsicum annum L.	Pepper	Hardwick 1965
	Datura ferox L.		
	D. stramonium L.	Jamestown weed	Capco 1957
	Lycopersicon esculentum	Tomato	Parsons 1939
			Parsons1939; 1940
			Capco 1957; Esguerra et al. 1969;
	Nicandra physalodes Gaertn	Apple of Peru	Parsons 1939; 1940
	Nicotilana tabacum L.	Tobacco	Capco 1957; Esguerra et al. 1969
			(Catan 1958); Parsons 1940;
			Hardwick 1965
	Physalis angulata L.		Parsons 1939; 1940
	P. peruviana L.	Cape gooseberry	Parsons 1940; Hardwick 1965
	Solanum melongena L.	Eggplant	Capco 1957
	S. nigrum	Sunberry	Parsons 1940
Umbelliferae			
	Daucus carota L.	Carrot	Hardwick 1965
Urticeae			
	Bochmeria nivea L.	Ramie	Baltazar 1968

Table 4. Natural enemies of *Helicoverpa*.

Natural Enemies	References
A. Parasitoids	
Trichogramma chilonis	Torreno, 1982; Torreno and Famoso, 1990; CRDI, 1985; Cadapan, 1986; Cacayorin et al. 1993;
Trichogramma chilotreae	Suharto, 1989; Cahatian, 1990; Pascua and Pascua, 1995
Trichogrammatoidea bactrae	Torreno, 1982; Torreno and Cadapan, 1984; Alba 1989; Famoso, 1990; Cadapan, 1986; Famoso and Alba, 1990
Trichogrammatoidea cojuancoi	Torreno and Cadapan, 1984; Alba, 1989; Famoso, 1990; Famoso and Alba 1990
Campoplex rufigastor	Pascua and Pascua, 1995
Rhopalida	Cahatian, 1990; Cacayorin et al. 1993.
Campomeris micans	Marcos, 1989
Eriborus sp.	Marcos, 1989
Campoletis sp.	Divina and Irabagon, 1976
Snellenius manilae	Cahatian, 1990; Solsoloy et al. 1994; Obien, 1987; Cacayorin et al., 1993
Brachymeria sp.	Cahatian, 1990
Trichomalopsis sp.	Cahatian, 1990
Enecorphilus sp.	Cahatian, 1990; Solsoloy et al., 1994; Cacayorin et al., 1993; Catan, 1958; Santhoy, 1980
	Santhoy, 1990
B. Predators	
Campyloma libida	
Cyrtopletis tenius	Suharto, 1990
Sphedanolestes mendicus	Marcos, 1989; James, 1988; Torreno 1990; Cahatian, 1990
Euagoras sp.	Marcos, 1989
Orius sp.	Marcos, 1989
Eumenes campaniformis	Catan, 1958
Eumenes pyriformis philippinensis	Baltazar, 1980
Rhyncium atrissimum	Baltazar, 1980
Salenopsis germinata	Cahatian, 1990
Eocanthecona furcellata	Cahatian, 1990; CRDI, 1985; Obien, 1987; Solsoloy et al., 1994
Tenodora sp.	Cahatian, 1991
Oxyopes javanus	Cahatian, 1991
	CRDI, 1985; Cacayorin et al., 1993
C. Pathogens	
Bacillus sp.	
Nomurea rileyi	Damo, 1988
	Damo, 1988

TOXIC EFFECTS OF QUININE FACTORY EFFLUENTS ON TILAPIA *OREOCHROMIS MOSSAMBICUS* AND AQUATIC ECOSYSTEM

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ABSTRACT

Three types of liquid wastes are produced in the quinine factory during extraction of quinidine from cinchona febrifuge. The wastes produced in the preliminary and interim phase of extraction were found to be highly acidic and that the waste produced in the final phase was alkaline. All the wastes contained thiocyanate and alkaloid residues and the interim phase waste, in addition, contained about 80% methanol. Bioassays with tilapia, Oreochromis mossambicus, showed that a very small concentration of the preliminary and interim phase wastes (0.0008 and 0.00062 ‰, respectively) reduced the feeding rate and growth of tilapia. High dose of the final phase waste (1.592 ‰) also reduced the feeding rate and yield of fish, but low to moderate dose (0.02 to 0.96 ‰) of this waste did not produce any adverse effect. Higher doses of preliminary (0.033-0.0716 ‰) and interim (0.007-0.011 ‰) phase waste individually or in combination with other waste drastically reduced the dissolved oxygen, primary productivity, phytoplankton and zooplankton population of water. Final phase waste reduced these parameters only at a dose of 1.592 ‰. Presence of both methanol and thiocyanate made the interim phase waste toxic. The critical lethal level of methanol in the interim phase waste was much lower than the critical concentration of pure methanol for fish. Even a small concentration of thiocyanate was found toxic if the liquid waste was acidic. The final phase waste containing a higher amount of alkaline thiocyanate produced less toxicity individually and in mixture condition. Complete removal of methanol and thiocyanate from the waste is recommended for hazardless disposal.

INTRODUCTION

Quinine and quinidine are two important alkaloids of cinchona which have many medicinal uses (Turner and Woodwards, 1953). A few factories in India produce these alkaloids in crude condition from the barks of cinchona. *Cinchona succirubra, C. officianalis, C. calisya,* by extraction with thiocyanate, methanol, strong alkali and oils (GOI, 1966). Residues of these reagents and alkaloids of cinchona were detected in moderate to high quantities in the liquid wastes from the Quinine Factory at Mungpo (Saha et al., 1988). Most of these residues are potentially toxic to animals and can cause serious damage to the receiving ecosystem if disposed without sufficient care.

We are not aware of any study on the chronic effects of liquid wastes from the quinine factory on aquatic organisms. Thiocyanate (Doudoroff, 1976; Watson and Maly, 1987; Heming et. al. 1985) and methanol (Poirier et al., 1986; Gluth et al., 1985), however, have been found to be toxic to aquatic organisms. The aim of this investigation is to assess the toxicity of liquid wastes from a quinine factory to fish and the aquatic ecosystem with reference to the thiocyanate and methanol content of the wastes.

MATERIALS AND METHODS

Liquid wastes

Three types of liquid wastes were collected from the factory. Procedure of collection and preservation of the wastes before their use have been described elsewhere (Saha et al., 1988). These wastes were marked as preliminary phase, interim phase and final phase wastes according to their steps of production in the factory (Fig. 1). pH, total solid, methanol, thiocyanate and cinchona alkaloid residues were estimated in the liquid wastes before their uses. Colour, ph and total solid of the liquid wastes were estimated according to the standard procedure described in APHA (1976). Methanol was estimated by fractional distillation and thiocyanate was estimated volumetrically by titration with 0.1 N $AgNO_3$ (Vogal, 1973). Important cinchona alkaloids left in the liquids were estimated by processing the solid residue of the liquid wastes (GOI, 1966).

Test organism

Tilapia *Oreochromis mossambicus* were procured from local farm and acclimatized to the test condition for 96-190h before their use. Adult tilapia of both sexes (mean total length 98±2 mm; mean weight 13.6 ± 0.5g) and fingerlings (mean total length 34 ± 2 mm; mean weight 0.56 ± 0.04g) were used in the experiments.

Bioassay

Bioassays were run with the liquid wastes in 15 l glass aquaria in the laboratory and 400 l outdoor cement tanks. Static 96 h bioassays were run in the laboratory to evaluate the effects of the liquid wastes on the feeding behaviour of fish. Each aquarium contained 10 l of unchlorinated tap water (temperature 24°C, pH 7, DO 6 mg/l, alkalinity 150 mg/l and hardness 100 mg/l). Only adult fish (two per aquarium) were used for the feeding test and at least five replicates were performed for each waste treatment. Altogether, there were 24 treatments, 4 for each of the individual waste and 12 of their mixtures tested (Table 1). Fish were given live earthworms daily at 0800 hr and were allowed to feed for 4 h. Unconsumed food was removed by siphoning to avoid any contamination. The amount of food consumed by the control fish was considered as 100%.

Survival, growth and reproduction of tilapia were assessed in outdoor 90-day static bioassays. Outdoor tanks were arranged in several blocks each with 4 tanks arranged in a Randomized Complete Block design (Gomez and Gomez, 1984). Each tank was provided with a 3-cm thick sediment at the bottom. After filling with about 400 l of tap water, plankton in each tank was allowed to grow naturally. When sufficient plankton had grown to serve as natural food of the fish, each tank was stocked with 15 fingerlings.

Each block of tanks was exposed to one sublethal concentration of either an individual liquid waste or mixture of the wastes (Table 1). Ten percent of the water in each tank was replaced weekly. For the supply of water for replacement, a separate set of tanks was maintained. In addition to the natural food, the stocked fish were fed 6 days a week with rice bran and mustard oil cake (1:1) at the rate of 10 percent of the total body weight of fish per day.

Dissolved oxygen, primary productivity and phytoplankton and zooplankton abundances were measured every 10 days during the outdoor bioassays (APHA, 1976). Fish were sampled at the end of the experiment (90d). Length, weight and visceral weight of all sampled fishes were recorded. Final biomass was used to estimate the yield of fish in each treatment. The formulae used to estimate the condition factor (K), visceral index (VI) and maturity index (MI) were adopted from LeCren (1951) and Bagenal (1978). This formulae were as follows:

$$K = \frac{\text{Body wt. (g)}}{\text{Body length (mm)}} \times 10^5; \quad VI = \frac{\text{Visceral wt.(g)}}{\text{Body wt. (g)}} \times 100; \quad MI = \frac{\text{Gonad wt.(g)}}{\text{Body wt.(g)}} \times 100$$

[where, K = condition factor; VI = visceral index; MI = maturity index]

Fecundity was estimated from the total number of ripening eggs per female.

Statistical Analysis

All data were statistically analyzed for significance of variance (ANOVA)and were evaluated at the 5% level of probability. Differences between the means were compared by Least Significant Difference (LSD) test or Duncan's Multiple Range Test (DMRT) according to the set of data (Gomez and Gomez, 1984).

RESULTS

Chemical Nature of Liquid Waste

The chemical nature of the liquid wastes was almost similar to that reported earlier by Saha et al.(1988) but the quantity of the various toxicants present in each liquid waste was different. The preliminary and interim phase liquid wastes were highly acidic (pH 4.5-4.6), but the final phase waste was slightly alkaline (pH 7.6). Thiocyanate was detected in all the wastes, but methanol was found only in the interim phase waste. Residues of the cinchona alkaloids were obtained from all the liquid wastes. The mean concentrations estimated from 100 ml of each waste for total solid, thiocyanate, methanol and cinchona alkaloid residues present in each of the treatments are shown in Table 2.

Feeding

The feeding behaviour of fish varied with concentration and chemical nature of the liquid wastes. Single factor ANOVA, followed by LSD test, showed that feeding rate of fish significantly reduced ($P<0.05$) at P_2-P_5 treatments (preliminary phase waste) and I_2-I_5 treatments (interim phase waste) as compared to control (Table 3). The P_5 and I_5 treatments induced a severe reduction in

the appetite of fish. But in the F_2-F_4 treatments (final phase liquid waste) feeding rate of fish significantly increased ($P<0.05$) over control. However, feeding rate significantly reduced ($P<0.05$) at F_5 treatment. Feeding rate of fish exposed to mixture of wastes were significantly reduced ($P<0.05$) in the M_2, M_3, M_6, M_7 and M_9 to M_{12} treatments. Feeding rate significantly increased ($P<0.05$) in the M_4, M_5 and M_{13} treatments (Table 4). In M_8 treatment, it was similar to ($P>0.05$).

The exposure containing higher concentration of either preliminary or interim phase waste individually or in combination with other waste acutely reduced the feeding rate of fish e.g. P_4-P_5, I_4-I_5, M_2, M_3, M_6, M_7, M_{10} - M_{12}, treatments. But feeding rate was stimulated by moderate doses of final phase waste (F_2-F_4 treatments) or by treatments containing final wastes along with small concentration of preliminary or interim phase wastes (i.e. M_5 and M_{13} treatments).

Survival and Growth of Fish

No mortality of fish was recorded in the control. However, 5-10% of the fish died with the various treatments of the liquid wastes in the outdoor tanks (Table 5). Yields of the fish were significantly reduced at P_2-P_5 treatments of preliminary phase waste, I_2-I_5 treatments of interim phase waste and F_5 treatment of final phase waste (Table 3). Yield of fish exposed to the mixture of wastes was significantly reduced at M_2, M_3, M_6-M_{12} treatment (Table 4). Yields were significantly increased at F_3 and F_4 treatments of final phase and at M_5 and M_{13} treatments of the mixture of wastes. High concentration of preliminary and interim phase waste reduced the yield either individually or in mixture condition irrespective of their combination e.g. P_5 I_5, M_2, M_3, M_6, M_9, M_{11}, and M_{12} treatments. Moderate concentration of final phase waste, on the contrary, stimulated the yield (e.g. F_3 and F_4 treatments). High concentration of final phase waste could counteract the ill effect of the interim phase waste when the latter was present in small concentration e.g. M_5, (I_2+F_5 treatments). At F_3, F_4, M_2, M_5 and M_{13} treatments, yields were significantly higher than control ($P<0.05$).

Frequency of size distribution showed significant variations among the treatments. Highest length of fish recorded at a significantly higher rate in F_3, F_4, M_5, and M_{13} treatments over that of the control (Table 5). Size groups recorded from control, F_2, M_4 and M_8 treatments were moderate and comparable to each other. In all other treatments, the growth was poor and significantly higher number of lower length groups were recorded as compared to control. From the comparison of length groups it appeared that low to moderate dose of final phase waste stimulated the growth of fish.

The condition factor (K) of fish was significantly reduced ($P<0.05$) in all the treatments as compared to that of the control (Tables 3 and 4). The visceral index (VI) was significantly reduced ($P<0.05$) in I_5, F_4, M_5, M_{10} and M_{13} treatments but increased significantly ($P<0.05$) in P_4, P_5, I_4, I_5, F_5, M_2, M_3, M_6, M_7, M_9, M_{11} and M_{12} treatments over control. The higher concentrations of the preliminary and interim phase wastes irrespective of their combinations increased the VI of the test fish.

Maturity Index

The maturity index (MI) of male tilapia significantly reduced ($P<0.05$) in P_4, P_5, I_4, I_5, M_2, M_3, M_6, M_7, M_9 - M_{12}, treatments (Tables 3 and 4). It increased with the F_3, F_4 and M_{13} treatments. With the other treatments, the MI was comparable with that of the control.

Fecundity significantly reduced ($P<0.05$) in P_3-P_5, I_3-I_5, F_5, M_2-M_3, M_6-M_{12} treatments. Fecundity increased in F_4 and M_{13} treatments (Tables 3 and 4). With the other treatments fecundity was comparable to control. Spawning was observed in all control tanks and among the following treated tanks P_2, I_2, F_2-F_4, M_5, M_7, M_8, M_{10} and M_{13}

Limnological Parameters

Changes in various limnological parameters during the outdoor bioassay have been shown in Figures 2-5. All parameters, after an initial increase up to 20-40 days, gradually decreased in all treatments. Gross primary productivity of water ranged from 95 to 219 mgC/m³/hr in control (P_1, I_1, F_1, M_1) while in P_5, I_5 and F_5 treatments it ranged respectively from 62 to 190, from 62 to 175 and from 61 to 187 mgC/m³/hr. Values of primary productivity in P_4 and I_4 treatments were also close respectively to P_5 and I_5 treatments and that of P_2-P_3, I_2-I_3 and F_2-F_4 were close to control. In mixtures, values of primary productivity in M_4, M_5 and M_{13} treatments were close to control, while in all other combinations the values were close to P_5 and I_5 treatments of individual wastes. The worst effects were produced by M_3 and M_9 treatments, both of which contained highest concentration of preliminary phase waste (P_5). Fluctuation in dissolved oxygen and phytoplankton densities among various treatments showed a trend similar to that of primary productivity in both individual and mixture treatments. Zooplankton populations were found more susceptible than phytoplankton to the highest dose of individual wastes particularly at P_5 and I_5 treatments. Under M_4, M_5, M_8, and M_{13} treatments of mixture, the phytoplankton densities were also comparable to control, while the zooplankton populations were comparable to only under M_5 and M_{13} treatments. In all other treatments of mixture the zooplankton populations were sharply decreased.

Significant variation (ANOVA) were observed in all the above parameters among various treatments of individual wastes and their mixtures (Table 6). Highest concentration of all the individual wastes (e.g. P_5, I_5, F_5 treatments) significantly reduced all the parameters throughout the study period. When the individual wastes were mixed together even the mixture containing a small concentration of the individual waste also significantly reduced these parameters (e.g. M_7 treatment).

DISCUSSION

The interim phase liquid waste contained about 80% methanol which was higher than the methanol proportion of the interim phase waste reported earlier (Saha et al., 1988). Thiocyanate content of this waste was also relatively higher in the present investigation. The pH of the final phase waste used in the present investigation was slightly alkaline (pH 7.6) in contrast to very high pH found earlier. Due to relatively low pH, a considerable amount of cinchona alkaloids were obtained in the present investigation.

Both methanol and thiocyanate are highly toxic to aquatic organisms. Thiocyanate has been found to inhibit ion transport across the gill of fish (Epstein et al., 1973; 1975) and reduce the appetite of fish (Eales and Shostak, 1983). Thiocyanate was found toxic to brook trout and rainbow trout at a concentration of 518 mg/l (Heming et al., 1985). Even a much lower concentration of thiocyanate in the preliminary phase waste (Table 2) reduced the appetite and growth of tilapia in the present study. Low pH of the preliminary phase waste probably increased the acuteness of thiocyanate toxicity (Watson and Maly, 1987).

The present study indicated that the toxic effect of thiocyanate could be reduced if the pH of the waste was increased. Thus, the final phase waste, which was alkaline, was found to be less toxic compared with the preliminary phase waste although it contained a high amount of thiocyanate. The low concentration of preliminary phase waste (P_3) which reduced the yield contained 0.03 mg/l thiocyanate. Since the preliminary phase waste did not contain any trace of methanol, it appeared that even 0.03 mg/l of thiocyanate could significantly reduce the growth of fish without any additive effect of methanol. However, the F_3 treatment of the final phase waste which had a concentration of thiocyanate which was 10 times higher (0.36 mg/l) did not affect any of the parameters tested, while almost similar concentration of thiocyanate (0.39 mg/l) in the P_5 treatment of preliminary phase waste produced a drastic effect on the growth of fish.

Thus, it was found that the concentration of thiocyanate which was harmless at the final phase waste could become lethal in the preliminary phase waste. Obviously, thiocyanate became more toxic with the acidic condition of the preliminary phase waste. Heming et al.(1985) observed that toxicity of thiocyanate to trout was most upredictable because of anomalous death and sudden appearance of many fatal symptoms designated as sudden death syndrome. It was stated that 50% of the trout would be at risk of sudden death syndrome when plasma concentration of thiocyanate reach approximately 250 mg/l. However, none of the symptoms enlisted by various authors (Garvin, 1939; Smith, 1973 and Heming et al., 1985) as sudden death syndrome could be detected in long term exposure of the liquid wastes in the present investigation. These symptoms were probably acute effects of thiocyanate and were exhibited at a high concentration of thiocyanate.

Chronic effects including the reduction in yield of fish is initiated at a much lower concentration. Only 0.01 mg/l thiocyanate in the P_2 treatment reduced the growth of fish. However, thiocyanate was not the only toxicant present in the wastes. Cinchona alkaloid residues probably also rendered some amount of toxicity to preliminary and interim phase wastes. Although information on the toxicity of cinchona alkaloids to aquatic organisms are little, many plant alkaloids are known to be potentially toxic to fish (Konar, 1969, 1970 and Jhingran, 1982).

Of the three liquid wastes, the interim phase waste was found most toxic because it contained 80% methanol which is a dangerous aquatic pollutant. Methanol is quickly absorbed in the body of fish from water (Gluth et al., 1985). Although wide ranges of data are available for the acute toxicity of pure methanol to fish (Weigelt et al., 1885; Powers, 1917 and Liebmann, 1960) a critical lethal concentration for fish has been estimated at 240 mg/l (Dawson et al., 1970). The 96h LC_{50} dose of interim phase waste for tilapia contained 0.0564 ml/l methanol (Saha et al., 1988) which was equivalent to 44.61 mg/l. The highest sublethal dose of interim phase waste used in the present investigation (I_3) contained 0.0088 ml/l methanol which was equivalent to 6.961 mg/l. Yields of fish were reduced even at the lowest concentration of interim phase waste tested (I_2). This dose contained only the minute concentration of methanol (0.0005 ml/l). Therefore, the critical lethal level of methanol present in the quinine factory waste was much lower than the critical lethal concentration of pure methanol estimated for fish. Moreover, interim phase waste contained both methanol and thiocyanate. Hence an additive toxicity of both was apprehended. Thus, when preliminary phase waste was combined with interim phase waste (M_2-M_4) further addition of thiocyanate from the preliminary phase waste increased the toxicity of the mixture. However, the toxic potential of the mixture depended upon the pH of the thiocyanate solution.

Thus, when low concentration of interim phase waste (low concentration of methanol) was mixed with high concentration of final phase waste (high concentration of alkaline thiocyanate) i.e. M_5 treatment, no significant change in the growth of fish was found as compared to control. Rather, yield of fish was increased. Primary productivity, phytoplankton and zooplankton populations in M_5 treatment were also comparable to control. M_6 and M_9 treatments also contained

high amount of alkaline thiocynate but there was drastic reduction in the yield of fish. M_6 contained high amount of interim phase waste and M_9 contained high amount of preliminary phase waste. M_{11} and M_{12} also contained high concentration of both preliminary and interim phase wastes and these treatments reduced the growth of fish.

Disastrous effects of the high dose of preliminary and interim phase wastes were also revealed from the marked reduction in dissolved oxygen, primary productivity, phytoplankton and zooplankton populations. The entire ecosystem was supposed to be choked by a dose of 0.0716 ‰ preliminary phase waste (P_5) or 0.011 ‰ of interim phase waste (I_5) or mixture containing any one of these wastes in such high concentration. Zooplankton population was found most affected. Sensitivity of crustacean zooplankton to these wastes has been recorded by Saha et. al., (1988). Present investigation indicated that, in addition to zooplankton, other parameters of aquatic ecosystem such as dissolved oxygen, primary productivity and phytoplankton populations were also alarmingly affected by high dose of preliminary and interim phase wastes.

Thus, it was indicated that although the final phase waste could reduce the toxicity of preliminary and interim phase wastes, the mixture containing higher doses of preliminary and interim phase wastes (i.e. containing high amount of methanol and acidic thiocyanate) remained potentially hazardous for fish and the aquatic ecosystem. The treatments of mixture that produced impacts similar to control (such as M_4, M_{10} and M_{13}) contained 0.04-0.12 mg/l thiocyanate and 0.0005 ml/l methanol. However, 0.11 mg/l thiocyanate at M_7 reduced the yield. Since such minute concentrations of thiocyanate and methanol could produce harmful effects in chronic bioassay, it can be concluded that they should be totally removed from the wastes before their disposal to avoid any adverse impact on the receiving aquatic ecosystem.

ACKNOWLEDGEMENTS

The authors are thankful to the Head, Department of Zoology of the University of Kalyani, for providing laboratory facilities. Grateful acknowledgements are also due to Dr. U. K. Som, Assistant Quinologist, Quinine Factory, Mungpo, West Bengal for providing many useful information.

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Table 1. Concentration of the liquid wastes and their combinations used during the experiment.

Dose of Individual Liquid Waste (‰, v/v)

Preliminary Phase Waste		Interim Phase Waste		Final Phase Waste	
Denoted as	Concentration	Denoted as	Concentration	Denoted as	Concentration
P_1	0.0000	I_1	0.00000	F_1	0.0000
P_2	0.0008	I_2	0.00062	F_2	0.0224
P_3	0.0052	I_3	0.00150	F_3	0.1028
P_4	0.0330	I_4	0.00710	F_4	0.9640
P_5	0.0716	I_5	0.01100	F_5	1.5920

Dose of Mixture Liquid Wastes (‰, v/v)

Denoted as	Combination	Preliminary Phase Waste	Interim Phase Waste	Preliminary Phase Waste
M_1	Control	0.0000	0.00000	0.0000
M_2	$P_2 + I_5$	0.0008	0.01100	0.0000
M_3	$P_5 + I_2$	0.0716	0.00062	0.0000
M_4	$P_2 + I_2$	0.0008	0.00062	0.0000
M_5	$I_2 + F_5$	0.0000	0.00062	1.5920
M_6	$I_5 + F_5$	0.0000	0.01100	1.5920
M_7	$I_2 + F_2$	0.0000	0.00062	0.0220
M_8	$P_2 + F_5$	0.0008	0.00000	1.5920
M_9	$P_5 + F_5$	0.0716	0.00000	1.5920
M_{10}	$P_2 + F_2$	0.0008	0.00000	0.0220
M_{11}	$P_2 + I_5 + F_5$	0.0008	0.01100	1.5920
M_{12}	$P_5 + I_2 + F_5$	0.0716	0.00062	1.5920
M_{13}	$P_2 + I_2 + F_2$	0.0008	0.00062	0.0220

Table 2. Concentration of total solid, thiocyanate, methanol and alkaloid residues in various treatments of the liquid wastes.

Treatment	Total Solid (mg/l)	Thiocyanate as NH_4SCN (mg/l)	Methanol (‰)	Alkaloid residues (ug/l)		
				Quinine	Cinchonidine	Cinchonine
			Preliminary Phase Waste			
(P_1)	0.00	0.00	0.0000	0.0	0.0	0.0
(P_2)	0.05	0.01	0.0000	1.9	1.5	3.3
(P_3)	0.33	0.03	0.0000	12.5	9.9	21.3
(P_4)	2.10	0.18	0.0000	79.2	62.7	135.9
(P_5)	4.55	0.39	0.0000	171.8	136.0	293.6
			Interim Phase Waste			
(I_1)	0.00	0.00	0.0000	0.0	0.0	0.0
(I_2)	0.12	0.03	0.0005	4.5	3.5	7.6
(I_3)	0.29	0.06	0.0012	10.8	8.5	18.4
(I_4)	1.35	0.29	0.0057	51.1	40.5	87.3
(I_5)	2.09	0.45	0.0088	79.2	62.7	135.3
			Final Phase Waste			
(F_1)	0.00	0.00	0.0000	0.0	0.0	0.0
(F_2)	0.28	0.08	0.0000	10.3	8.1	17.7
(F_3)	1.26	0.36	0.0000	47.3	37.0	81.2
(F_4)	11.86	3.37	0.0000	443.4	347.0	761.5
(F_5)	19.58	5.57	0.0000	732.3	573.1	257.7

Table 3. Effects of individual liquid wastes on the feeding rate, yield, condition factor (K), visceral index (VI), fecundity and maturity index (MI) of tilapia. Data are mean of four replicates. Least Significant difference (LSD, P<0.05) between two means are indicated by different letters.

Treatment	Food Consumed (%)	Yield (Kg/ha)	K	VI	Fecundity	MI (Male)	MI (Female)
			Preliminary Phase Waste				
P_1	100.00^a	1700^a	5.70^a	9.53^d	202^a	0.32^a	1.82^a
P_2	71.82^b	1456^b	3.95^b	9.17^e	195^a	0.21^b	1.62^b
P_3	64.95^b	1302^c	3.30^c	9.86^c	151^b	0.20^b	1.41^c
P_4	36.08^c	854^d	3.88^b	11.05^b	97^c	0.13^b	1.05^d
P_5	17.52^d	570^e	3.41^c	11.60^a	86^d	0.11^b	0.64^e
			Interim Phase Waste				
I_1	100.00^a	1700^a	5.70^b	9.53^c	202^a	0.32^a	1.82^a
I_2	76.63^b	1570^b	2.99^b	9.02^d	204^a	0.24^a	1.29^b
I_3	73.20^b	1370^c	3.57^b	8.85^d	158^b	0.22^a	1.27^b
I_4	46.39^c	1021^d	3.08^c	10.91^b	108^c	0.18^b	1.06^c
I_5	18.56^d	540^e	0.72^a	12.74^a	71^d	0.09^b	0.32^d
			Final Phase Waste				
F_1	100.00^a	1700^c	5.70^a	9.53^b	202^c	0.32^b	1.82^c
F_2	110.91^a	1695^c	2.99^c	9.51^b	193^c	0.27^b	1.98^b
F_3	120.96^b	1900^b	3.57^b	9.69^b	212^b	0.55^a	3.16^a
F_4	128.95^a	2227^a	3.08^c	6.16^c	229^a	0.51^a	3.21^a
F_5	82.39^c	1250^d	0.72^d	10.81^a	145^d	0.22^b	1.32^d

Table 4. Effects of mixture of liquid wastes on the feeding rate, yield, condition factor (K), visceral index (VI), fecundity and maturity index (MI) of tilapia. Data are mean of four replicates. Results of DMR test has been represented by small letters. Common letters between any two treatment means of a column indicates their similarity while two different letters indicate significant difference at 5% level.

Treatment	Food Consumed (%)	Yield (Kg/ha)	K	VI	Fecundity	MI (Male)	MI (Female)
			MIXTURE LIQUID WASTES				
M_1	100.00^{c}	1843^{b}	3.07^{b}	9.53^{f}	207.50^{b}	0.38^{c}	2.04^{b}
M_2	39.18^{fg}	934^{fg}	1.30^{g}	13.15^{b}	81.75^{ef}	0.10^{g}	0.43^{f}
M_3	45.02^{ef}	1134^{de}	1.45^{f}	12.35^{c}	86.75^{e}	0.12^{fg}	0.44^{f}
M_4	110.05^{b}	1960^{b}	2.84^{d}	9.53^{f}	202.00^{b}	0.41^{bc}	1.35^{d}
M_5	139.55^{a}	2322^{a}	2.93^{c}	7.52^{i}	216.25^{a}	0.48^{ab}	3.16^{a}
M_6	36.67^{gh}	851^{g}	1.00^{j}	12.52^{c}	85.00^{e}	0.14^{efg}	0.52^{f}
M_7	23.78^{hi}	635^{h}	0.84^{k}	11.64^{d}	75.25^{fg}	0.11^{fg}	0.28^{g}
M_8	93.89^{d}	1657^{c}	0.83^{d}	9.12^{g}	165.75^{c}	0.29^{d}	1.56^{c}
M_9	65.33^{e}	662^{h}	1.23^{h}	14.04^{a}	75.00^{g}	0.13^{efg}	0.43^{f}
M_{10}	20.44^{i}	1208^{d}	1.07^{i}	8.74^{h}	116.25^{d}	0.20^{e}	1.26^{d}
M_{11}	41.24^{ef}	1067^{e}	1.81^{e}	11.24^{e}	80.00^{efg}	0.12^{fg}	0.86^{e}
M_{12}	42.53^{ef}	1031^{ef}	1.82^{e}	11.65^{d}	81.00^{efg}	0.18^{ef}	0.91^{e}
M_{13}	132.73^{a}	2441^{a}	4.15^{a}	6.82^{j}	219.50^{a}	0.54^{a}	3.23^{a}

Table 5. Survival and frequency of various size groups recorded under various treatments.

Treatment	Number stocked	% of total survived	% of various size groups (mm) recorded				
			(70-79)	(60-69)	(50-59)	(40-49)	(30-39)
			Preliminary Phase Waste				
P_1	60	100	00	60	40	00	00
P_2	60	97	00	19	81	00	00
P_3	60	83	00	14	77	09	00
P_4	60	87	00	00	29	65	06
P_5	60	83	00	00	00	58	42
			Interim Phase Waste				
I_1	60	100	00	60	40	00	00
I_2	60	97	00	40	60	00	00
I_3	60	92	00	25	75	00	00
I_4	60	88	00	00	72	28	00
I_5	60	80	00	00	06	94	00
			Final Phase Waste				
F_1	60	100	00	60	40	00	00
F_2	60	93	00	64	36	00	00
F_3	60	95	12	56	32	00	00
F_4	60	95	20	59	21	00	00
F_5	60	85	00	43	57	00	00
			MIXTURE LIQUID WASTES				
M_1	60	100	00	65	35	00	00
M_2	60	87	00	00	73	27	00
M_3	60	82	00	20	66	14	00
M_4	60	100	05	67	28	00	00
M_5	60	93	25	65	10	00	00
M_6	60	87	00	00	62	38	00
M_7	60	81	00	00	27	73	00
M_8	60	95	00	60	40	00	00
M_9	60	82	00	00	41	59	00
M_{10}	60	87	00	27	71	02	00
M_{11}	60	85	00	15	63	22	00
M_{12}	60	85	00	22	57	21	00
M_{13}	60	97	19	78	03	00	00

Table 6. Results of ANOVA of various limnological parameters among various treatments of various wastes.

Names of Wastes	Sources of Variation	df	F values			
			DO	PP	Zooplankton Population	Phytoplankton Population
Preliminary Phase Waste	Treatment	4				
	Error	12	22.38	47.22	373.61	60.73
Interim Phase Waste	Treatment	4				
	Error	12	60.73	37.22	147.21	14701.82
Final Phase Waste	Treatment	4				
	Error	12	130.28	109.33	192.39	11454.19
Mixture Liquid Waste	Treatment	12				
	Error	36	15.09	83.97	132.92	26423.00

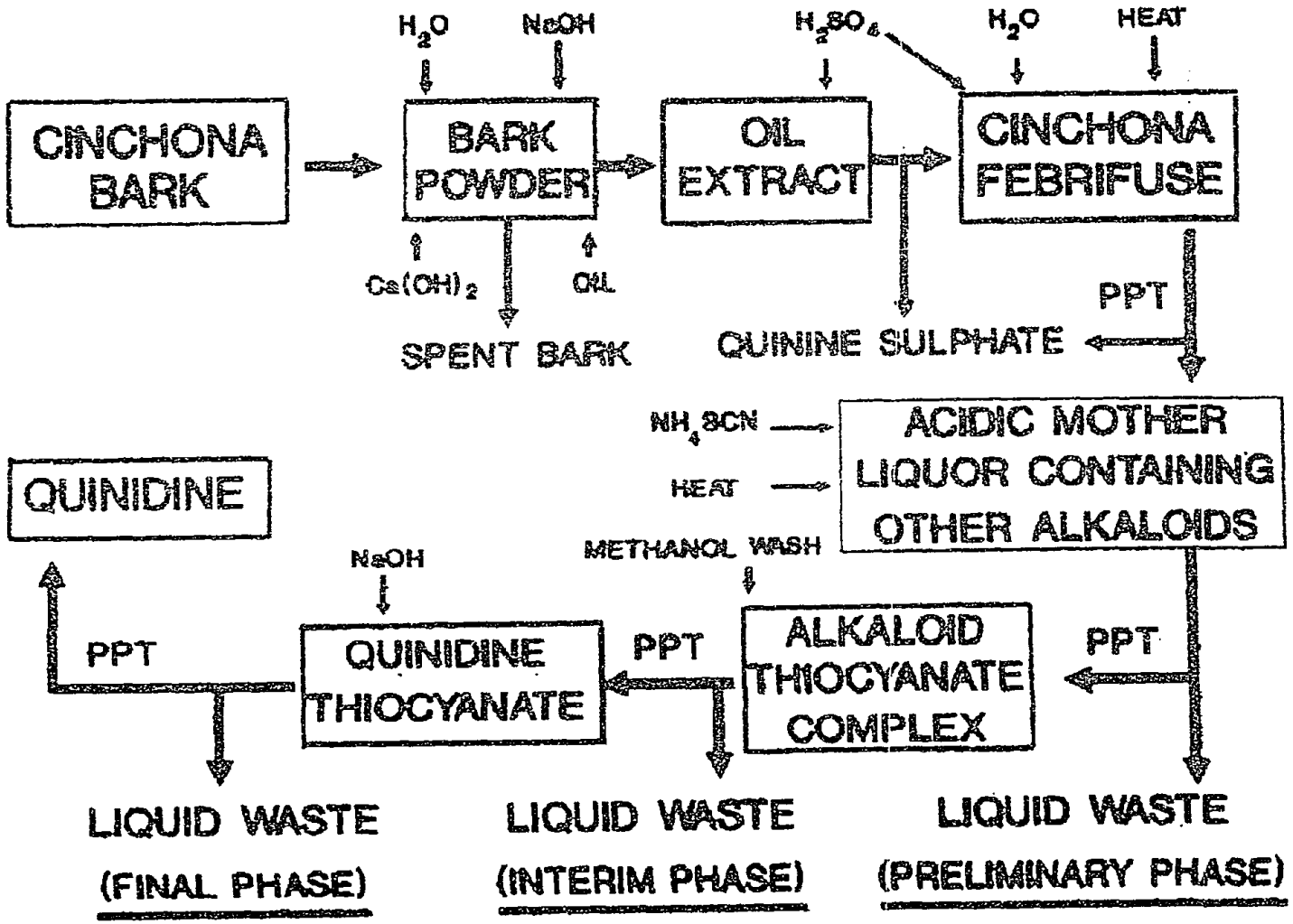

Figure 1. An outline scheme of quinine and quinidine extraction from cinchona bark and production of the liquid wastes.

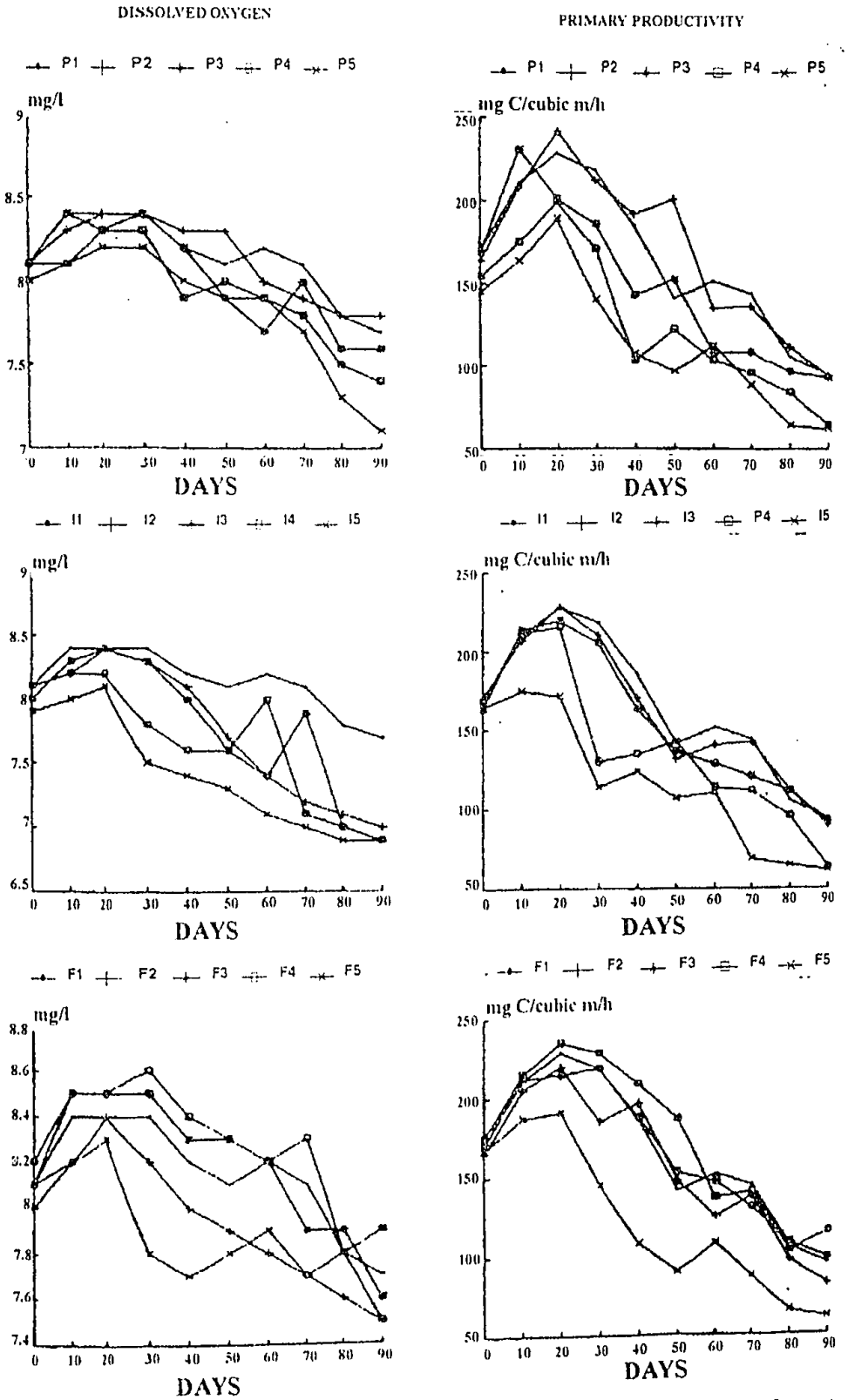

Figure 2. Variation of dissolved oxygen and primary productivity in control and treatments of individual liquid wastes. (Legends for treatments have been given in Table 1).

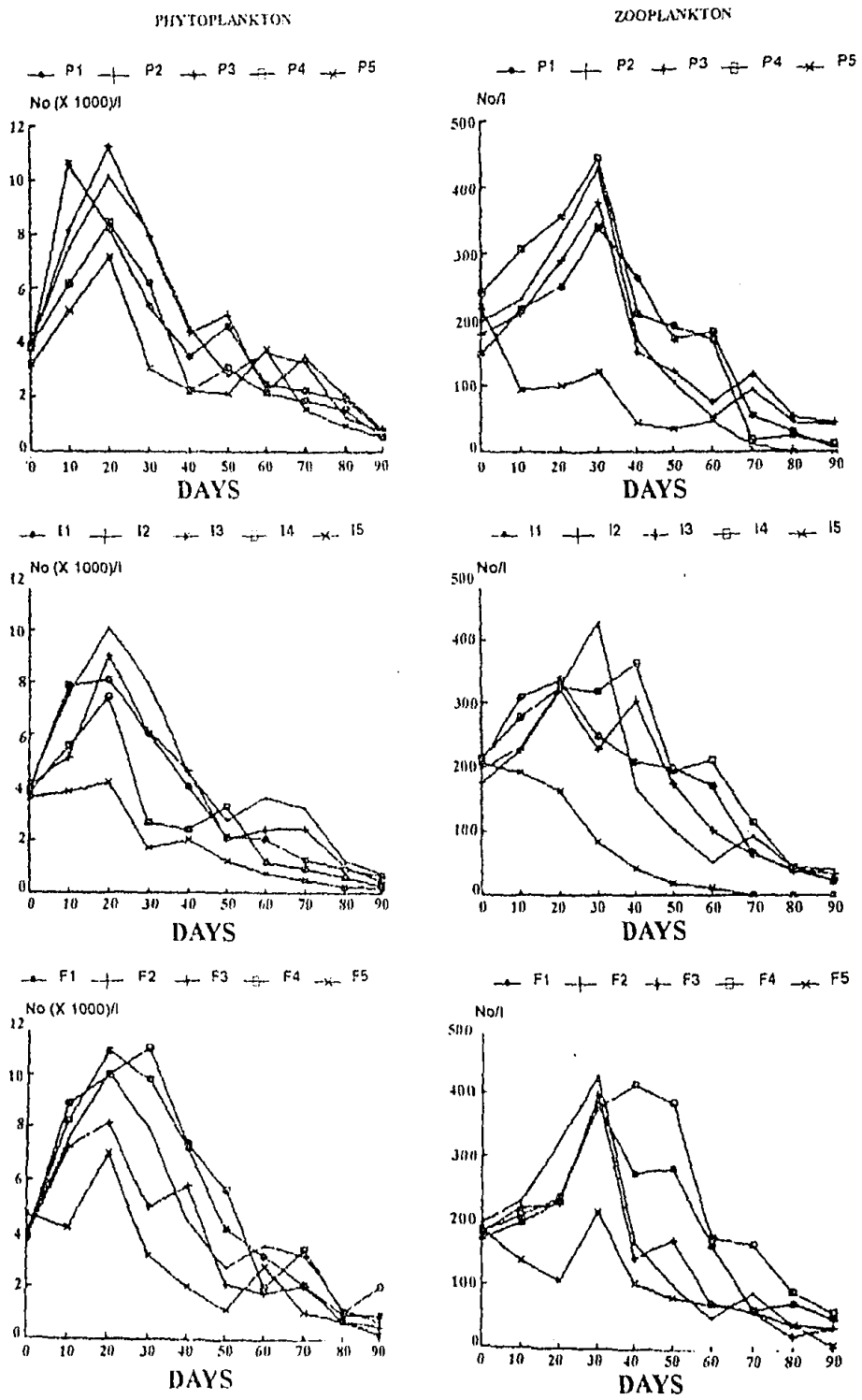

Figure 3. Phytoplankton and zooplankton abundances in control and treatments of individual liquid wastes.

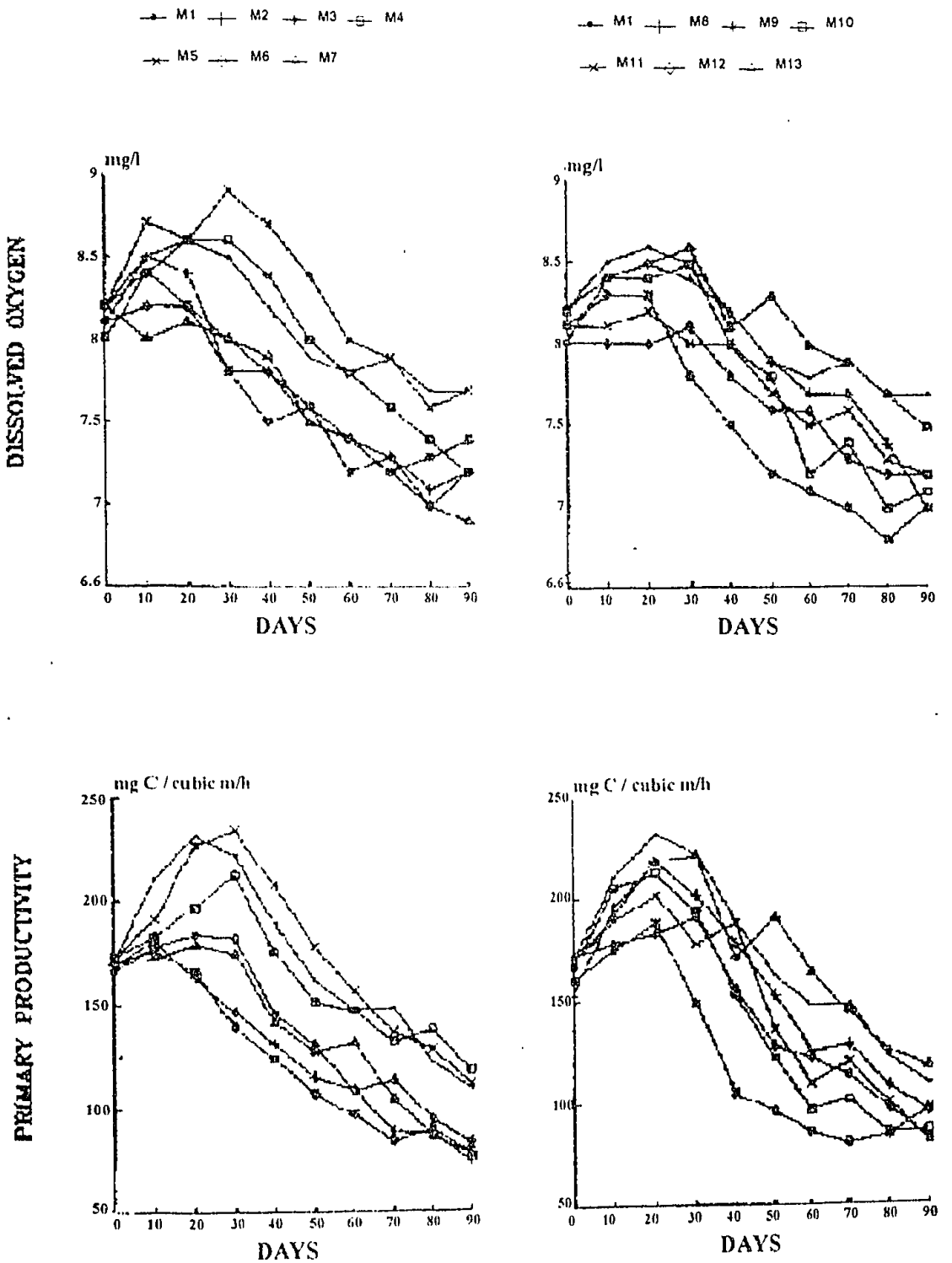

Figure 4. Variation of dissolved oxygen and primary productivity in control and treatments of mixture of liquid wastes. (Legends for treatments have been given in Table 1).

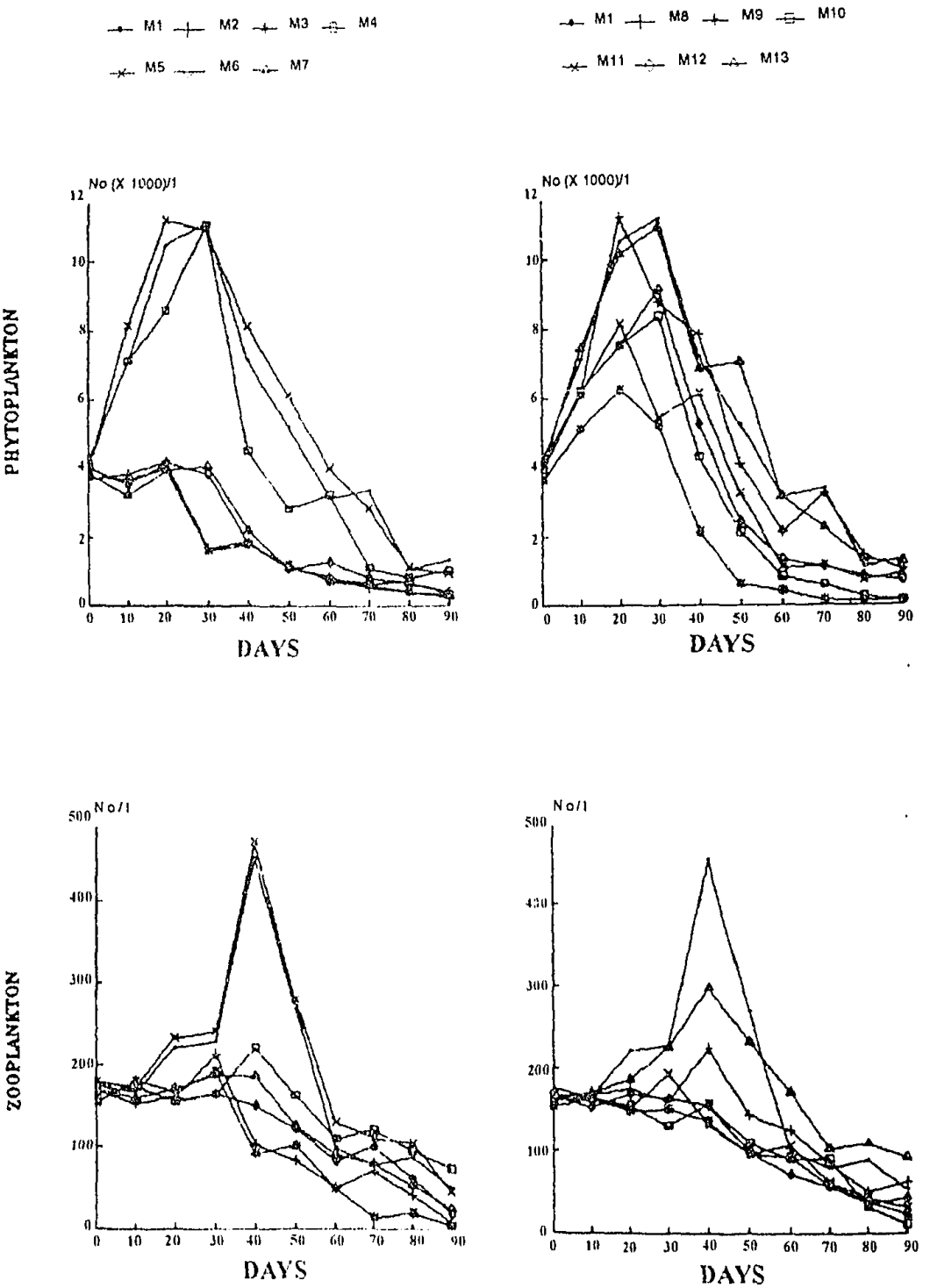

Figure 5. Variation of phytoplankton and zooplankton abundances in control and treatments of mixture liquid wastes.

A BIOACTIVE CAROTENOID FROM *MIMOSA INVISA*

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Keywords: *Mimosa invisa*, Makahiyang lalake, Leguminosae, lutein, carotenoid, antimutagen, antimicrobial, cytotoxic

ABSTRACT

Four isolates were obtained from the chloroform extract of the air dried leaves of Mimosa invisa by vacuum liquid and gravity column chromatography (dry packing). Their cytotoxicity were evaluated by the brine shrimp assay. The LC_{50} of isolates 1, 2, 3 and 4 were 416, 272, 24.2 and 281 µg/mL, respectively. Based on LC_{50}, 3 showed significant antitumor and anticancer potential. Thus, further biological activity tests were conducted on 3. Micronucleus test revealed that 3 at a dosage of 0.200 mg/kg reduced the number of micronucleated polychromatic erythrocytes (MPCE) induced by Mitomycin C by 81%, indicating that it is an antimutagen. Isolate 3 of concentrations 0.5, 0.7, 1.0, 1.4 and 2.0 µg/mL was further tested for antimicrobial potential by the disc diffusion method. It showed maximum activity at 2.0 µg/mL against the following bacteria: Bacillus subtilis, Staphylococcus aureus, Escherichia coli and Shigella dysenteriae and fungus: Candida albicans. The structure of 3 was elucidated by extensive 1D and 2D NMR and UV spectroscopy. It was identified as lutein, a widely distributed carotenoid.

INTRODUCTION

This study was conducted to isolate any bioactive constituents that may be present in *Mimosa invisa* or Makahiyang lalake (leguminosae), a common weed found throughout the Philippines. To date, there have been very few studies conducted on *M. invisa* and none of these studies reported on the pharmacological activities of this plant. Previous studies on *M. invisa* reported the presence of alkaloid in the leaves, seeds and roots (Douglas, 1957) and afzelia oils (Gunstone, 1971) and lectin (Chandrika et al., 1987) from the seeds. Studies on the congeners of the weed reported the isolation of chalcones from the leaves of *M. temuefolia* (Garcia et al., 1989)] saponins from the bark of *M. acmiflora* (Anton 1991) and tubulin from *M. pudica* (Mukherjee et

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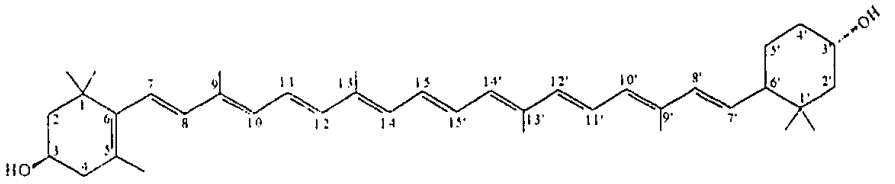


al., 1982). We now report the isolation and structure determination of lutein (3) and its cytotoxicity, antimicrobial and antimutagenicity potentials.

A previous study on carotenoids reported that lutein has anticancer and antioxidant properties (Gester, 1993). A number of studies on the anticancer properties of related carotenoids have been reported (Bendich, 1994; Bertran, 1994; Nishino, 1994; Underwood, 1985; Santamaria et al., 1988). In addition, antimutagenicity studies by Ames test have been conducted on cathaxanthin, ß-carotene, 8'-apo-3-carotenal and 8-apo-ß-carotene (Azuini ett al., 1992). However, this is the first report on the cytotoxicity, antimutagenicity and antimicrobial activities of lutein.

RESULTS AND DISCUSSION

Four isolates were obtained from the chloroform extract of *M. invisa* by gravity column and vacuum liquid chromatography. The isolates were evaluated for their cytotoxicity by the brine shrimp test. Results of the study shown in Table 1 indicated that **3** has the highest cytotoxicity with 100% death of nuplii at a concentration of 1 mg/mL. The LC_{50} value was determined using probit analysis (Finney, 1971). Isolates having LC_{50} less than 30 µg/mL are considered bioactive following protocols established by the National Cancer Institute (Meyer, 1982). The LC_{50} for isolates **1**, **2**, **3** and **4** are 416, 272, 24.2 and 281 µg/mL, respectively. Based on LC_{50}, **3** showed significant antitumor and anticancer potential, while isolates **1**, **2** and **4** were found inactive.

Since **3** showed promising bioactivity, it was further tested for antimicrobial potential. As shown in Table 2, isolate **3** has antibacterial activity against *B. subtilis*, *S. aureus*, *E. coli* and *S. dysenteriae*. The first three microorganisms are the common pathogens found in pus cells. Thus, **3** could be used to prevent the growth of these microorganisms in open wounds and other infected tissues. It was also observed that this isolate has an antifungal potential against *C. albicans*, the fungus that causes sore throat and some gastrointestinal disorders.

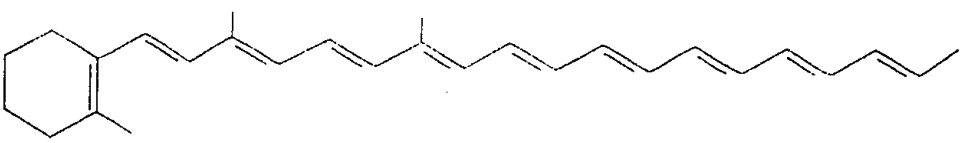
To further test the activity of **3** in animal system, its antimutagenicity potential was evaluated by the Micronucleus test. It was observed that **3** is an antimutagen, since at a dosage of 0.200 mg/kg mouse, it reduced the number of MPCE that was induced by Mitomycin C (Table 3). A significant reduction of 81% suggests that this bioactive isolate could be used to prevent the proliferation of tumor cells in animal systems.

The structure of **3** was determined by extensive 1D and 2D NMR and UV spectroscopy as follows:

The ^{1}H-NMR spectral data (Table 4) revealed the presence of ten methyl singlets, six of which were allylic as indicated by their downfield resonances at δ 1.61 (3H, s), 1.72 (3H, s), 1.90 (3H, s) and 1.95 (9H, s). The integrals revealed thirteen overlapping olefinic protons at δ 6.14 (5H, m), 6.23 (2H, d, br), 6.32 (1H, s), 6.37 (1H, s) and 6.61 (4H, m) and two additional olefinic protons at δ 5.53 (1H, s, br) and 5.45 (1H, dd). Two carbinyl hydrogens were attributed to the resonances at δ 4.24 (1H, s, br) and 4.0 (1H, s, br).

The ^{13}C-NMR spectrum (Table 4) showed thirty-six carbons. However, a total of forty carbons may be accounted for by four types of overlapping carbons deduced as follows: The downfield resonances at δ 124.5 to 138.5 showed nineteen olefinic carbons, three of which were composed of two overlapping carbons at δ 137.8, 137.6 and 132.6 as indicated by their large absorption intensities. Thus, the olefinic region afforded twenty-two carbons. Whereas, ^{1}H NMR revealed ten methyl groups, ^{13}C NMR showed overlapping carbons of two methyl groups at δ 12.84 (the absorption intensity was almost twice that of the other methyl carbons). Two carbons singly bonded to oxygens were deduced from the resonances at δ 65.2 and 66.0. The DEPT spectral data (Table 4) indicated three methylene carbons at δ 42.6, 44.7 and 48.5; one methine carbon at δ 55.0 and two oxygenated methine carbons at δ 65.2 and 66.0; two quaternary carbons at δ 34.1 and 37.2; seven nonprotonated olefinic carbons at δ 124.9, 126.2, 135.1, 135.7, 136.45, 136.53 and 138 and fifteen olefinic methine carbons at 124.5, 125.0, 125.7, 128.8, 130.1, 130.14, 130.9, 131.3, 132.6 (2C), 137.6 (2C), 137.8 (2C) and 138.5. The ^{1}H and ^{13}C assignments for 3 were verified by the heteronuclear 2D experiment HMQC which indicated the hydrogens directly bonded to carbons.

Isolate 3 is an orange crystal which showed λ_{max} at 445 and 474 nm (EtOH). These suggested the presence of conjugated double bonds characteristics of carotenoids. Comparison of the λ_{max} of 3 with those of carotenoids found in the literature (Goodwin, 1976) indicated that 3 may have the following chromophore:



The Fisher-Kuhn rules (Silverstein et al., 1981) gave a calculated λ_{max} for the above chromophore as 447.5 nm which is close to the observed λ_{max} of 445 nm for 3. To fully elucidate the structure of 3, further NMR spectral data were obtained.

The COSY spectrum showed the following isolated spin systems. The olefinic proton at δ 6.1 (H8') is coupled to another olefinic proton at δ 5.4 (H7'), which is further coupled to the hydrogen at δ 2.4 (H6'). The latter hydrogen is coupled to the olefinic proton at δ 5.5 (H4'), which is in turn coupled to a CH_3 at δ 1.61 (allylic methyl) and a carbinyl hydrogen at δ 4.2 (H3'), which is finally coupled to the methylene protons at δ 1.40 (H2'a) and 1.90 (H2'b). This coupling chain is shown in Fig. 1. Another isolated spin system is indicated by the resonance at δ 4.0 which is coupled to four methylene protons at δ 1.45 (H2a), 1.80 (H2b), 2.0 (H4a) and 2.40 (H4b). Fig. 2 shows the second fragment of 3. Coupling between the olefinic protons at the deshielded region (δ 6.1 - 6.6) and allylic methyl groups at δ 1.72 - 1.95 were observed, but isolated spin systems could not be deduced due to overlapping resonances. However, allylic couplings were observed between the methyl groups at δ 1.90 (3H, s) and 1.95 (9H, s) and the deshielded protons at δ 6.1.

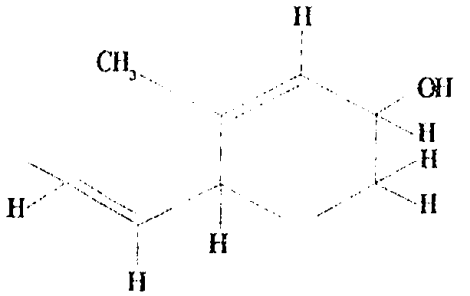


Fig. 1. Fragment 1

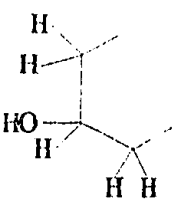


Fig. 2. Fragment 2

From the proposed chromophore based on Fisher-Kuhn rules, fragment 2 becomes part of ring A, while fragment 1 becomes part of ring B. To confirm the proposed structure, HMBC spectrum which is an inverse long-range heteronuclear experiment optimized for J = 8 Hz was obtained. Thus, the carbon at δ 55 (C6') is long-range correlated to the methyl protons at δ 1.61 (allylic), 0.97 (aliphatic) and 0.84 (aliphatic) and the olefinic protons at δ 5.5 (H4') and 6.1 (H8'). The carbon at 44.7 is long-range correlated to the methyl protons at 0.84 (aliphatic) and 0.97 (aliphatic), while the one at δ 66 is long-range correlated to the methylene protons at δ 1.40 (H2a') and 1.90 (H2b'). Additional long-range correlations were observed between the carbon at δ 124.5 and the protons at δ 1.61 (allylic CH_3), 1.90 (H2a'), 1.40 (H2b') and 2.40 (H6'). The carbon at δ 138 is long-range correlated to the protons at δ 2.40 (H6'), 1.61 (allylic CH_3) and 5.4 (H7'), while the one at δ 55 is long-range correlated to the protons at δ 5.5 (H4'), 6.10 (H8'), 1.61 (allylic CH_3), 0.84 (aliphatic CH_3) and 0.97 (aliphatic CH_3). Further correlations were observed between the carbon at δ 34.1 and the protons at δ 0.84 (aliphatic CH3), 0.97 (aliphatic CH_3), 2.40 (H6') and 1.40 (H2'), and the carbon at δ 128 and the protons at δ 2.40 (H6') and 6.1 (H8'). Long-range correlations were also observed between the carbon at δ 37.2 and the protons at δ 1.06 (aliphatic CH_3). Also correlated were the carbon at δ 65.2 and the protons at δ 1.45 (H2a) and 2.0 (H4a), and the carbon at δ 42.6 and the protons at δ 1.72 (allylic CH_3) and 1.45 (H2a). Long-range correlations were likewise observed between the carbon at δ 124.9 and the protons at δ 2.40 (H4b), 1.72 (allylic CH_3) and 2.0 (H4a), and the carbon at δ 136.5 and the protons at 1.06 (aliphatic CH_3), 1.72 (allylic CH_3), 1.80 (H2b) and 1.45 (H2a). Long-range correlations were also observed between the olefinic carbons at the deshielded regions (δ 124.9 - 137.6) and the four methyl groups at the deshielded region (δ 1.90 - 1.95). Table 5 gives a summary of the HMBC spectral data. All long-range correlations observed were consistent with the structure of 3.

Literature search revealed that 3 is lutein, a widespread catorenoid. Confirmatory evidence was the ^{13}C NMR spectrum of 3 and lutein found in the literature (Goodwin, 1976). The spectra matched in all essential respects, thus 3 is lutein. Lutein was also isolated by our research group from *Azadirachta indica*, *Cosmos caudatus*, *Vernonia cinerea* and an *Alternanthera* sp.

EXPERIMENTAL

General

The ^1H nmr spectrum of isolate 3 in $CDCl_3$ was recorded with the use of Bruker AMX 300, while Hitachi UV-VIS spectrophotometer was used for recording the UV spectrum of isolate 3 in ethanol. Fractions were monitored by TLC and spots were visualized by spraying with vanillin/ H_2SO_4, then warming.

Sample Collection

Mimosa invisa was collected from a wasteland in Lupon, Davao Oriental in September 1995. It was identified as *Mimosa invisa* at the National Museum.

Extraction and Isolation

Air dried leaves (500 g) were osterized and soaked in chloroform (4 L) to give a crude extract (46.5 g). The extract (10 g) was subjected to vacuum liquid chromatography (VLC) packed with silica gel (60G) with increasing proportions of EtOAc in petroleum ether (10% increment) as eluent,

followed by increasing proportions of MeOH in EtOAc (10% increment) up to 30% MeOH in EtOAc and finally MeOH. Fifteen fractions (100 mL) were collected. Fractions 1-3 gave isolate **1** [30 mg, R_f = 0.45 (10% EtOAc in petroleum ether)] after rechromatography (gravity column) in 10% EtOAc in petroleum ether. Fractions 5-6 gave isolate **2** [26 mg, R_f = 0.52 (40% EtOAc in petroleum ether)] after rechromatography (gravity column) in 40% EtOAc in petroleum ether. Fractions 8-10 gave isolate **3** [60 mg, R_f = 0.62, 80% EtOAc in petroleum ether)] after rechromatography (gravity column) in 80% EtOAc in petroleum ether. Fractions 11-13 gave isolate **4** [25 mg, R_f = 0.45 (EtOAc)] after rechromatography (gravity column) in EtOAc.

Bioassay

A. Cytotoxicity Test

The four isolates were evaluated for their cytotoxicity by the brine shrimp test. *Artemisia salina* eggs (brine shrimp eggs) were used as test organism for cytotoxicity test (Meyer et al., 1982). These eggs were hatched in a rectangular hatchery tank. Ten shrimps were transferred to each vial, then seawater was added to make 5 mL. This discs containing **1-4** (10, 100, 1000 mg/mL) were placed in vials. Twelve replicates were prepared for each dose level. Survivors were counted after 24 hours with the aid of a magnifying glass and the % death for each dose and control were determined. The LC_{50} was determined using probit analysis (Finney, 1971).

B. Micronucleus Test

Mitomycin C (2.75 mg/kg mouse) and isolate **3** (0.200 mg/kg mouse) dissolved in dimethylsulfoxide (DMSO) (7.5 mL/kg mouse) were administered orally to mice of the Swiss strain (7-12 weeks from DOST). For the control, only the mutagen, Mitomycin C (positive control) and DMSO (solvent control) were administered orally to the same strain of mice. For each isolate and control, three mice were tested. The second administration was carried out after twenty-four hours. Six hours after the second administration, the mice were sacrificed and blood from the bone marrow was flushed with fetal calf serum. Blood from the bone marrow was smeared on slides, three per mouse were prepared. The slides were stained with undiluted May Gruenwald solution, followed by 50% May Gruenwald solution, then 15% Giemsa stain (Schmid, 1976). The number of micronucleated polychromatic erythrocytes (MPCE) per 1000 polychromatic erythrocytes (PCE) were then counted with the use of a high power microscope, and results are given as % reduction in MPCE.

C. Antimicrobial Test

The test bacteria (clinical isolation) used were *Salmonella typhi*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Vibrio cholera*, *Shigella dysenteriae* and *Pseudomonas aeruginosa*, while the test fungi were *Candida albicans*, *Aspergillus niger* and *Saccharomyces cerevisiae*. Concentrations of 0.5, 0.7, 1.0, 1.4 and 2.0 µg/mL of isolate **3** were used. The petri dishes were incubated at 37°C and evaluated for antimicrobial activity by measuring the diameter of the inhibition zones after 24 hr. for bacteria and 48 hr. for fungi.

CONCLUSION

A bioactive carotenoid, which was identified as lutein (**3**), was isolated from the chloroform extract of the air-dried leaves of *Mimosa invisa*. Its LC_{50} of 24 indicated that it has antitumor and anticancer potential. This was supported by the Micronucleus test which revealed that at a dosage

of 0.200 mg/kg, 3 reduced the number of MPCE induced by Mitomycin C by 81%. Compound 3 at a concentration of 2 µg/mL was also found to inhibit the growth of *B. subtilis*, *S. aureus*, *E. coli*, *S. dysenteriae* and *C. albicans*. This is the first report on the antimicrobial activity of 3.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the research grant from the Department of Science and Technology-Science Education Institute and the technical assistance of Jessie Sandro of the University of San Carlos.

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Table 1. Brine Shrimp Bioassay Data of Isolates 1-4.

Isolate	% Death Nauplii[a]			
	10 µg/mL	100 µg/mL	1000 µg/mL	LC_{50}[b] µg/mL
1	7.5	46.7	86.7	416
2	10.8	69.2	85.3	272
3	8.3	99.2	100	24.2
4	20	53.3	93.3	281

[a]determined from 12 replicates, methanol for control which has zero % death nauplii
[b]calculated using probit analysis

Table 2. Antimicrobial Bioassay Data of 3.

Microorganism	Zone of Inhibition (mm)[a]									
	After 24 hr.					After 48 hr.				
	0.5 µg/mL	0.7 µg/mL	1.0 µg/mL	1.4 µg/mL	2.0 µg/mL	0.5 µg/mL	0.7 µg/mL	1.0 µg/mL	1.4 µg/mL	2.0 µg/mL
S. typhi	-	-	-	T	T					
B. subtilis	9.5	11.5	16.1	18.2	23.1					
S. aureus	9.1	11.5	14.1	16.4	18.5					
E. coli	9.8	12.1	15.0	17.0	19.5					
V. cholera	-	-	-	-	T					
S. dysenteriae	10.0	13.0	16.6	18.5	21.5					
S. aeruginosa	-	-	-	-	T					
S. cerevisae						-	-	-	-	-
S. niger						-	-	-	-	-
C. albicans						11.5	13.9	16.5	18.5	22.0

[a]average diameter of 9 filter discs (8-mm diameter), chloroform used as control which showed no significant inhibition zones
T: Thinning
-: No zone inhibition

Table 3. Micronucleus Test Results of 3.

Sample Dosage (mg/kg mouse)	Average No. of MPCE/1000PCE (per slide) ± σ[a]	Percent Reduction (%)
0.200	5.22 ± 0.66	81.28%
(+) Control	17.6 ± 0.52	
(-) Control	2.6 ± 0.52	

[a]determined from 9 slides.

Table 4. 300 MHz ^1H, ^{13}C, DEPT and HMQC NMR Spectral Data of 3 in $CDCl_3$.

Carbons	$^{13}C,\delta$	$^1H,\delta$	Functionalities
1	37.2	-	-C-
2	48.5	1.45, 1.80	CH_2
3	65.2	4.0	CH-OH
4	42.6	2.0, 2.4	CH_2
5	124.9	-	CH=
6	136.5	-	CH=
1'	34.1	-	-C-
2'	44.7	1.40, 1.90	CH_2
3'	66	4.20	CH-OH
4'	125	5.5	CH=
5'	138	-	-C=
6'	55	2.40	CH
7'	128.8	5.4	CH=
allylic CH_3	12.78	1.95 (3H, s)	CH_3
allylic CH_3	12.84	1.95 (6H, s)	2 CH_3
allylic CH_3	13.1	1.90 (3H, s)	CH_3
allylic CH_3	21.6	1.72 (3H, s)	CH_3
allylic CH_3	22.9	1.61 (3H, s)	CH_3
ring B CH_3	24.4	0.84 (3H, s)	CH_3
ring B CH_3	28.8	0.97 (3H, s)	CH_3
ring A CH_3's	29.5, 30.3	1.06 (6H, s)	2 CH_3
	124.9, 126.2, 135.1, 135.7, 136.45, 136.53, 138.0	-	7-CH=
	124.5, 130.1, 130.14, 130.9	6.6	4 CH=
	125.7, 131.3, 137.8 (2C), 138.5	6.1	5 CH=
	132.6 (2C)	6.2	2 CH=
	137.6 (2C)	6.3, 6.4	2 CH=

Table 5. Long-Range ^{1}H - ^{13}C Correction (HMBC) Spectral Data of 3 in $CDCl_3$.

Carbon	^{13}C, δ	Long-range het. corr. expt.
C1	37.2	2 CH_3, H2
C2	48.5	2 CH_3
C3	62.5	H2a, H4a
C4	42.6	allylic CH_3, H2a
C5	124.9	allylic CH_3, H4a, H4b
C6	136.5	allylic CH_3, 2 CH_3, H2a, H2b
C1'	34.1	2 CH_3, H2', H6'
C2'	44.7	2 CH_3
C3'	66	H2a', H2b'
C4'	124.5	allylic CH_3, H2a', H2b', H6
C5'	138.0	allylic CH_3, H6', H7'
C6'	55.0	allylic CH_3, 2 CH_3, H4', H8'
C7'	128.0	H6', H8'

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milliliter	ml
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Energy and Work	KJ
kilojoule (replace calorie in dietetics)	
Mass	
kilogram	kg
gram	g
ton (metric ton)	t
milligram	mg
Time (same units used in both Metric and English System)	
day	d
hour	h
minute	min
second	s
Amount of substance	
mode	mole
Temperature	
degree celsius	^oC